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(54) Reagents and method for detecting polychlorinated biphenyls.

(57) Reagents and an immunoassay for detecting the presence or amount of polychlorinated biphenyls in a test sample. The assay is performed by adding a known concentration of a tracer labeled with a detectable moiety and a known concentration of an analyte-specific antibody to a test sample to form a mixture, incubating the mixture to form labeled tracer-antibody and analyte-antibody complexes, and determining the presence or amount of tracer-antibody complexes formed as a measure of the presence or amount of analyte in the test sample. Reagents provided include tracers, immunogens and an additive compound useful in preventing non-specific binding of the polychlorinated biphenyls to proteins which may be present in the test sample. A kit for performing the assay also is provided.

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BACKGROUND OF THE INVENTION

This invention relates generally to polychlorinated biphenyls, and more particularly, relates to reagents and an immunoassay for detecting the presence or amount of polychlorinated biphenyls in a test sample.

Current Environmental Protection Agency (EPA) approved methodology (EPA method 608, SW 846 method 8080) for determining the presence or amount of polychlorinated biphenyls (PCBs) in a test sample involves extracting the sample with an organic solvent such as hexane or methylene chloride, and then using a gas chromatograph (GC) equipped with an electron capture detector (ECD)to analyze the extract. A more rigorous pretreatment of test sample involving washing with sulfuric acid, mercury desulfurization, or purification on magnesium silicate (Florosil®, available from Aldrich Chem. Co., Milwaukee, WI) or alumina sometimes is required. The problems encountered with this methodology are inherent to the analytical technique: the procedure is time-consuming and expensive since the GC/ECD runs only one sample at a time, requires 40-60 minutes per test sample, and highly trained technical personnel are required to perform the testing and maintain the equipment.

Various radioimmunoassays (RIAs) for detecting PCBs have been reported. For example, a radioimmunoassay for detecting the commercial PCB mixtures aroclor 1242,1248 and 1254 has been reported in M. I. Luster, *et al.*, *Toxicology and Applied Pharmacology*, 1979, *50*, 147-155. In this method, antisera raised in rabbits using three immunogens, haptens 4-amino-4'-chlorobiphenyl, 2-amino-4,5,3',4'-tetrachlorobiphenyl, and 3-amino-2,6,2',6'-tetrachlorobiphenyl were linked *via* an adipamide linker arm to bovine serum albumin (BSA) and thyroglobulin, and ^{125}I tracers used in the method were prepared from the 5-bromovaleramide derivatives of the haptens. Minimum sensitivity of 1-3 ng was reported on standard samples. Feasibility was shown for detecting mixtures of these aroclors in mineral oil.

The detection of PCBs in milk and blood by radioimmunoassay is reported in W. H. Newsome and J. B. Shields, *Intern. J. Environ. Anal. Chem.*,1981, *10*, 295-304. The hapten employed in this assay was 2-amino-2',4,4',5,5'-pentachlorobiphenyl.Antisera was raised in rabbits using a succinamide linking arm to the hapten and the radiotracer was 2-[^{125}iodo]-2',4,4',5,5'-pentachlorobiphenyl. The minimum sensitivity reported was 0.1ng for aroclor 1260. The sensitivity reported for aroclor 1254 was similar, but lower aroclors were not detected with the same sensitivity.

U. S. Patent 4,456,691 to S. Stark teaches the preparation of polyclonal antibodies to PCBs using aroclor 1254 which has been aminated, diazotized and coupled to Bovine Serum Albumin (BSA). The antisera was evaluated by an RIA.

Radioimmunoassays are known to provide sensitive results. However, these assays usually require a higher degree of technical expertise, are more cumbersome that other immunoassay methods, require expensive equipment and involve the handling of radioactive materials.

It would be advantageous to provide an assay which could be used to detect aroclors 1221, 1232, 1242, 1016, 1248, 1254 and 1260 in single assay. It also would be advantageous to provide an assay which did not employ the use of radioisotopes. It further would be advantageous to provide an assay which utilizes a similar sample preparation to that of the EPA method, but can detect the presence or amount of PCBs in the test sample more rapidly and in an automated system.

SUMMARY OF THE INVENTION

The present invention provides a method for detecting the presence or amount of an analyte comprising polychlorinated biphenyls (PCBs) in a test sample. The method comprises the steps of adding a known concentration of a tracer labeled with a detectable moiety and a known concentration of an analyte-specific antibody to a test sample to form a mixture, incubating said mixture under conditions and for a time sufficient to form labeled tracer-antibody and analyte-antibody complexes, and determining the presence or amount of labeled tracer-antibody complexes formed as a measure of the presence or amount of analyte in the test sample. An extraction step may be performed on the test sample. The preferred detectable moiety is fluorescein or a fluorescein derivative. Complexes formed are measured by a fluorescent polarization immunoassay. The method also provides a step wherein a compound is contacted with the mixture to diminish non-specific binding of PCBs and PCB tracer to protein.

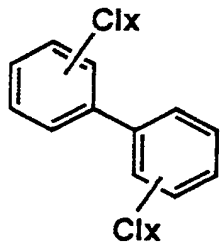
Also, the present invention provides hapten compounds, tracer compounds which are used as reagents in the method, immunogen compounds used to raise antibodies for use as reagents in the method, compounds useful for diminishing the non-specific binding of PCBs and the PCB tracer, and a kit for use in the method.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are applicable to the present invention: The term "determinants", as used herein, refers to those regions of the antigen which are involved in specific binding reactions between antigens and antibodies. In essence, it is the determinants which differentiate antigens, and therefore antibodies, from one another on the basis of immunological specificity.

The term "test sample", as used herein, refers to a sample to be tested for the presence or amount of the analyte of interest. The sample may be in a liquid or solid form, and include soil samples, oil samples, water samples, and other samples which are described in EPA methodology 608, SW 846 method 8080, which is incorporated in its entirety by reference.

The term "analyte", as used herein, refers to a molecule to which a binding member such as an antibody can be obtained or formed. The analyte of interest in the present invention is the class of compounds which are polychlorinated biphenyls of the following structure:



where x = 0-5, thus comprising a group of related congeners from mono to decachlorobiphenyl. Such an analyte is a protein-free compound of low molecular weight, generally in the range of from about 50 to about 4000 daltons, more preferably in the range of from about 100 to about 2000 daltons. Such an analyte does not induce antibody formation when injected into an animal but is reactive with antibodies.

The term "analyte-analog", as used herein , refers to a molecule which has substantially the same spatial and polar organization as one or more determinates of the analyte of interest. Such an analyte-analog is a protein-free compound, of low molecular weight, generally in the range of from about 50 to about 4000 daltons, more preferably in the range of from about 100 to about 2000 daltons. This duplication of the determinant(s) enables the analyte-analog to compete with the analyte in the test sample for a binding site on an analyte-specific binding member, such as an antibody. In addition, the analyte-analog can be modified such that it is not identical to the analyte while retaining the necessary determinant(s) for binding to an analyte-specific binding member.

The structure of the analyte-analog determinant(s) need not be identical to that of the analyte; it is sufficient that the analyte-analog substantially duplicate the appropriate determinant(s). Therefore, the analyte-analog can be any molecular structure which contains chemical groups, amino acids, or nucleotides different from those of the analyte, so long as that member (i.e., antibody, receptor, nucleotide sequence, etc.) will recognize and bind to that substantially duplicated determinant(s).

The term "analyte-specific binding member", as used herein, refers to a member, such as an antibody or a receptor, that specifically binds to the analyte. Antibodies, either polyclonal or monoclonal, to such an analyte typically are raised by first conjugating the analyte or analyte-analog to a protein carrier and then injecting the conjugate into an animal. The resulting antibodies can be isolated by conventional, well-known antibody isolation techniques.

The term "tracer", as used herein, refers to an analyte or an analyte-analog which is labeled with a detectable moiety, described hereinafter. The detectable moiety is the signal producing component of the tracer.

In accordance with the method of the present invention, a test sample suspected of containing an analyte of interest is mixed with a labeled tracer and an antibody specific for the analyte and the tracer and incubated. The test sample may be prepared by following EPA-approved extraction methodologies, such as extracting the sample with an organic solvent such as hexane or methylene chloride, as described in EPA method 608, SW 846 method 8080. Any analyte present in the sample and the tracer compete for a limited number of binding sites on the antibody, resulting in the formation of analyte-antibody and tracer-antibody complexes. By maintaining constant the concentration of tracer and antibody, the ratio of the formation of analyte-antibody complex to tracer-antibody complex is directly proportional to the amount of analyte present in the sample.

The preferred detection method is by fluorescence polarization. In this method, the amount of analyte in the sample is determined by exciting the mixture with polarized light and measuring the polarization of the

fluorescence emitted by free tracer and tracer-antibody complex. A tracer which is not complexed to an antibody is free to rotate in less than the time required for adsorption and re-emission of fluorescent light. As a result, the re-emitted light is relatively randomly orientated so that the fluorescence polarization of a tracer not complexed to an antibody is low, approaching zero. Upon complexing with a specific antibody, the tracer antibody complex thus formed assumes the rotation of the antibody molecule, which is slower than that of the relatively small tracer molecule, thereby increasing the polarization observed. Therefore, when an analyte competes with the tracer for antibody sites, the observed polarization of fluorescence of the tracer-antibody complex becomes a value somewhere between that of the free tracer and the tracer-antibody complex. If the sample contains a high concentration of the analyte, the observed polarization value is closer to that of the free tracer, i.e., low. If the sample contains a low concentration of the analyte, the polarization value is closer to that of the bound tracer, i.e., high. By sequentially exciting the reaction mixture of an immunoassay with vertically and then horizontally polarized light, and analyzing only the vertical component of the emitted light, the polarization of the fluorescence in the reaction mixture can be accurately determined. The precise relationship between polarization and concentration of the analyte to be determined is established by measuring the polarization values of calibrators having known concentrations. The concentration of the analyte can be interpolated from a standard curve prepared in this manner.

The immunoassay according to the invention is referred to as a homogeneous assay, which means that the final polarization readings are taken from a solution in which bound tracer is not separated from free tracer. This is a distinct advantage over heterogeneous immunoassay procedures, wherein the bound tracer must be separated from the free tracer before a reading can be taken.

Decectable moieties such as chemiluminescent molecules, luminescent molecules, enzymes, and other detectable moieties known to those of ordinary skill in the art may be used in the performance of the invention. In the present invention, the preferred detectable moieties are the luminescent molecules fluorescein and fluorescein derivatives. The choice of the fluorescent molecule for labeling the analyte-analog and thereby forming the tracer is advantageously flexible and is based substantially on the preferences of the routineer. It will readily be appreciated that the fluorescent labels are ideally chosen in accordance with their size, that is, the smaller the molecule, the more rapid it will be able to rotate, and thus the more effective it will be as an fluorescence polarization immunoassay (FPIA) tracer component. These compounds provide fluorescent response when excited by polarized light of an appropriate wavelength and thereby enable the fluorescence polarization measurement. Examples of fluorescein derivatives which can be used in the present invention include fluorescein amine, carboxyfluorescein, α-iodoacetamidofluorescein, 4'-aminomethyl-fluorescein, 4'-N-alkylaminomethylfluorescein, 5-aminomethylfluorescein, 6-aminomethyl-fluorescein, 2,4-dichloro-1,3,5-triazin-2-yl-aminofluorescein (DTAF), 4-chloro-6-methoxy-1,3,5-triazin-2-yl-aminofluorescein, fluorescein isothiocyanate. Especially preferred derivatives are aminomethylfluorescein and 5-carboxyfluorescein.

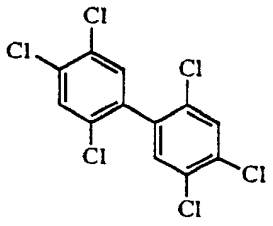
Fluorescein exists in two tautomeric forms depending on the acid concentration (pH) of the environment. In the open (acid) form, fluorescein or a fluorescein derivative (or a tracer containing a fluorescent molecule) is capable of absorbing blue light and emitting green fluorescence after an excited state lifetime of about four nanoseconds. When the open and closed forms coexist, relative concentration of molecules in the open and closed forms is easily altered by adjustment of the pH level. Generally, the tracers of the present invention are prepared in solution as biologically acceptable salts such as sodium, potassium, ammonium and the like, which allows the compounds to exist in the open, fluorescent form. The specific salt present will depend on the buffer used to adjust the pH level. For example, in the presence of sodium phosphate buffer, the compounds of the present invention will generally exist in the open form, as a sodium salt.

As used herein, the term "fluorescein", either as an individual compound or as a component of a tracer, is meant to include both the open and closed tautomeric forms, if they exist for a particular molecule, except in the context of fluorescence, in which case an open form is necessary for the fluorescence to occur.

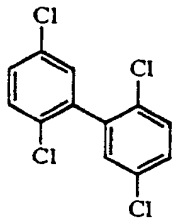
The particular tracers formed in accordance with this invention have been found to produce good assay results, as will be demonstrated in the detailed examples. The concentration of the analyte which can be determined in accordance with the present invention is from about 10^{-6} to about 10^{-10}M. Higher concentration of analyte can be determined by diluting the test sample. Although the concentration range of analyte in the sample will determine the range of concentration of the test reagents such as tracer and antibody, the individual reagent concentrations are determined empirically to optimize the sensitivity of the assay. Suitable concentrations of the tracer and antibody can be ascertained by one of ordinary skill in the art.

Haptens which are structurally similar to polychlorinated biphenyls are prepared for use as immunogens

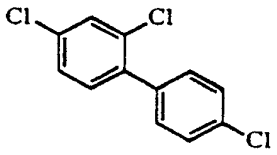
to raise antibodies, and/or analyte-analogs of tracers. Since there are 209 congeners possible in the family of polychlorinated biphenyls, haptens were chosen to resemble selected PCB congeners of the structures below:



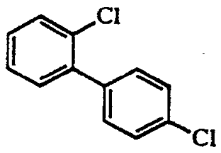
2,2',4,4',5,5'- hexachlorobiphenyl



2,2',5,5'- tetrachlorobiphenyl



2,4,4' -trichlorobiphenyl

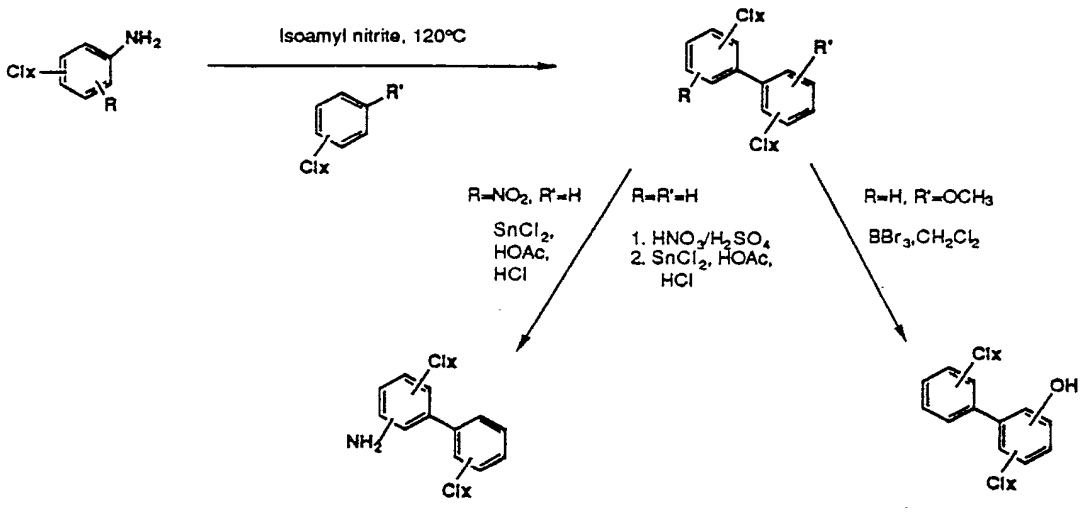


2,4'- dichlorobiphenyl

These are four congeners of some of the more prominent congeners present in the commercial aroclor mixtures. It is contemplated that one of ordinary skill in the art may select other prominent congeners from the family of PCBs on which to base an assay which would behave similarly.

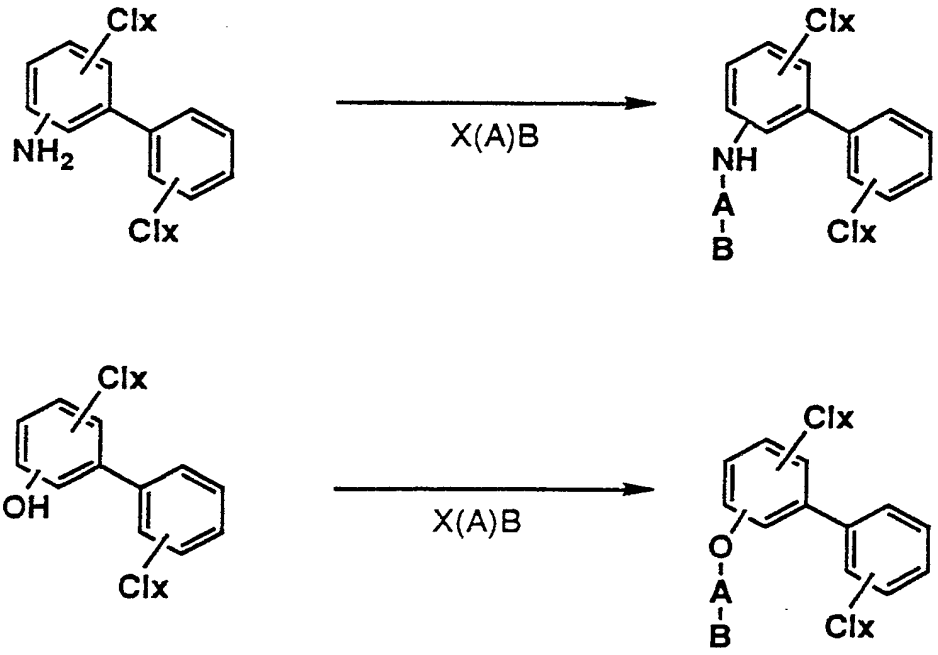
Haptens were prepared to mimic the congeners above by replacing a chloro group with either an amino or hydroxyl group. Both substituted groups have similar size to the chloro group and maintain a lone pair of electrons similar to the chloro group. The amino or hydroxy substituted PCB congeners were prepared by standard methods according to the scheme (I).

SCHEME I:



Linker arms were added to the amino or hydroxy substituted PCB congeners according to the scheme (II):

SCHEME II

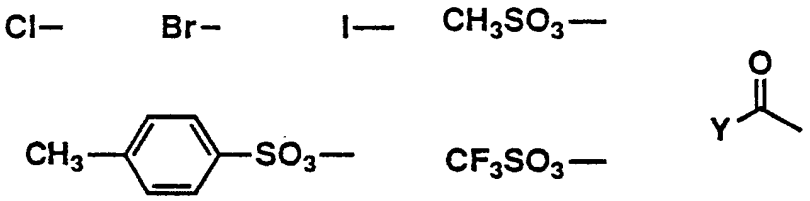


wherein A is a spacer group consisting of from 0 to 50 carbon atoms and heteroatoms, including not more than ten heteroatoms, arranged in a straight or branched chain, saturated or unsaturated, with the proviso that not more than two heteroatoms may be linked in sequence and that branchings may occur only on carbon atoms;

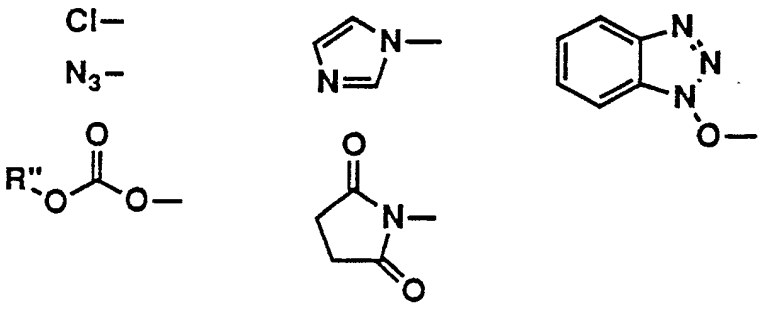
and wherein B is a linking group selected from

—CO_2H
—NH_2
—CHO
—OH

and wherein X is a reactive group selected from



and wherein Y is a carboxyl activating group chosen from

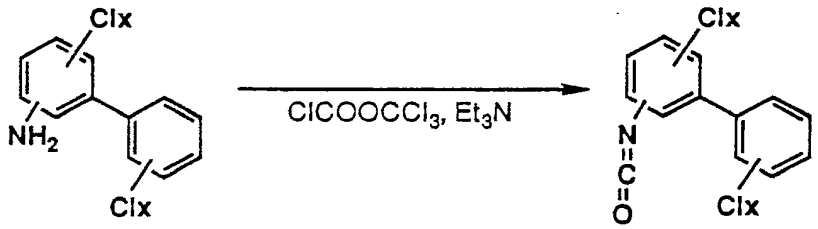


or any other recognized carboxyl activating group known to one of ordinary skill in the art.

When A involves only carbon atoms, it is preferred that A is from 1 to 10 carbon atoms. Suitable heteroatoms include nitrogen, oxygen, sulfur and phosphorus. For example, where A includes nitrogen and oxygen, A could be $-CH_2CH=N-O-CH_2-$. It appears that compounds with more than two heteroatoms in sequence are less stable.

Alternatively, the amino substituted PCB congeners may be activated directly according to scheme (III).

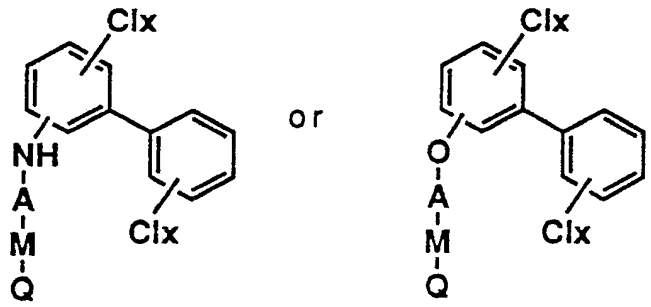
SCHEME III



Haptens are prepared according to methods known to those skilled in the art to produce compounds with a side chain containing chemical groups substantially similar to those of the desired determinant(s). These compounds, or their derivatives, are then attached to either a poly(amino acid) carrier or fluorescent molecule.

The antibodies utilized in the present invention were prepared by developing a response in an animal to one to the immunogens described hereinafter. The immunogen was administered and the appropriate antibodies were selected according to methods well-known to those skilled in the art. Although rabbits and mice were the immune hosts used in the experiments described herein, any *in vivo* host capable of producing antibodies to the immunogens can be used. The antibodies bind with PCBs present in the test sample as well as with the tracer.

Immunogens can be produced from a wide variety of PCB derivatives. The immunogens of the present invention have one of the following general structures:



wherein A is a spacer group consisting of from 0 to 50 carbon atoms and heteroatoms, including not more than ten heteroatoms, arranged in a straight or branched chain, saturated or unsaturated, with the proviso that not more than two heteroatoms may be linked in sequence and that branchings may occur only on carbon atoms;

and wherein M is a linking group selected from >C=O, -NH-, O-C=O, N-C=O, N-C=S; and Q is an immunogenic carrier.

A variety of protein carriers can be used as the poly(amino acid) immunogenic carrier. Suitable immunogenic carriers include albumins, serum proteins (e.g., globulins), ocular lens proteins, lipoproteins, and the like. Illustrative protein carriers are BSA, keyhole limpet hemocyanin (KLH), egg ovalbumin, thyroglobulin, and bovine gamma globulin. Alternatively, a suitable derivatized lipopolysaccharide (LPS) or synthetic poly(amino acid) may be utilized.

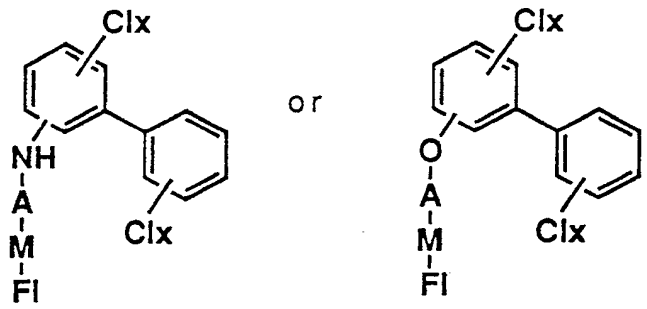
In the immunogens of the present invention, the chemical bonds between the carboxyl group containing PCB haptens and the amino groups on a protein carrier can be established using a variety of methods known to those skilled in the art. It is frequently preferable to form amide bonds. Amide bonds are formed by first activating the carboxylic acid moiety of the PCB hapten by reaction with an activating reagent such as 1,3-dicyclohexylcarbodiimide and an additive such as N-hydroxysuccinimide. The activated form of the hapten is then reacted with a buffered solution containing the carrier protein. Alternatively, the carboxylic acid hapten may be converted, with or without isolation, into a highly reactive mixed anhydride, acyl halide, acyl imidazolide, or mixed carbonate and then combined with the carrier protein. One of ordinary skill in the art will realize that there are many reagents that can be used to form amide bounds other than those listed.

A PCB hapten with a terminal amine functionality can be transformed into a highly reactive N-hydroxysuccinimide urethane by reaction with N,N'-disuccinimidyl carbonate in a suitable solvent, such as acetonitrile or dimethylformamide. The resultant urethane is then reacted with the carrier protein in a buffered, aqueous solution to provide an immunogen.

A PCB hapten with a terminal aldehyde functionality can be coupled to the carrier protein in a buffered, aqueous solution and in the presence of sodium cyanoborohydride, by reductive amination according the methods known to those skilled in the art.

Alternatively, a PCB hapten containing an alcohol group can be coupled to the carrier protein by first reacting it with phosgene or a phosgene equivalent, such as di- or triphosgene or carbonyldiimidazole, resulting in the formation of a highly reactive chloroformate or imidazoloformate derivative (usually without isolation). The resultant active formate ester is then reacted with the carrier protein in a buffered, aqueous solution to provide an immunogen.

Preferred tracers of the present invention can be produced from a variety of PCB derivatives and have the general structure:



wherein A is a spacer group consisting of from 0 to 50 carbon atoms and heteroatoms, including not more than ten heteroatoms, arranged in a straight or branched chain, saturated or unsaturated, with the proviso that not more than two heteroatoms may be linked in sequence and that branchings may occur only on carbon atoms; and

wherein M is a linking group selected from >C=O, -NH-, O-C=O, N-C=O, N-C=S; and

wherein Fl is a detectible moiety, preferably fluorescein or a fluorescein derivative.

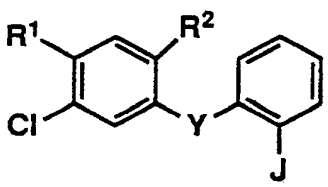
In the present invention it is preferred that taken together groups A and M consist of 0 to 12 carbon atoms and heteroatoms as described above.

A PCB hapten containing either an amino group or a carboxyl group can be coupled to fluorescein or a fluorescein derivative to prepare the tracers of the present invention. A PCB hapten with a terminal carboxyl group can be coupled to an amino-terminal fluorescein derivative by first activating the carboxylic acid moiety of the PCB hapten by reaction with an activating reagent such as 1,3-dicyclohexylcarbodiimide and an additive such as N-hydroxysuccinimide. The activated form of the hapten is then reacted with a solution of the fluorescein derivative, resulting in the formation of a tracer. Alternatively, the carboxylic acid hapten may be converted, with or without isolation, into a highly reactive mixed anhydride, acyl halide, acyl imidazolide, or mixed carbonate and then combined with the carrier protein. One of ordinary skill in the art will recognize that there are many reagents that can be used to form amide bonds other than those listed.

A PCB hapten with a terminal amine functionality can be transformed into a highly reactive N-hydroxysuccinimide urethane by reaction with N,N'-disuccinimidyl carbonate in a suitable solvent, such as acetonitrile or dimethylformamide. Or, according to Scheme (III), an amino PCB hapten can be activated to an isocyanate. The resultant products are then reacted with an amino fluorescein derivative to form urea tracers. An amino group containing hapten can also be coupled to a carboxyfluorescein derivative which has been activated with N-hydroxysuccinimide in a suitable solvent.

Alternatively, a PCB hapten containing an alcohol group can be coupled to the carrier protein by first reacting it with phosgene or a phosgene equivalent, such as di-or triphosgene or carbonyldiimidazole, resulting in the formation of a highly reactive chloroformate or imidazoloformate derivative (usually without isolation). The resultant active formate ester is then reacted with an amino-terminal fluorescein derivative resulting the formation of a tracer.

Additives which serve to decrease non-specific interaction of PCBs, PCB tracers, and analyte-analogs with proteins, surfaces and the like, include such substances as detergents, organic solvents, and other compounds of the general structure:



wherein Y is O or NH and one of R^1 and R^2 is chloro and the other is hydrogen, and J is CO_2H or CH_2CO_2H.

The compound preferred for reducing non-specific binding of PCBs and tracers to proteins and surfaces in the present invention is fenclofenac as taught by K. E. Godfrey, U. S. Patent No. 3, 766, 263. Although this compound has been employed as an agent to displace thyroid hormones from serum binding

proteins, its use in assays for PCBs is not heretofore known. [D.C. Atkinson, et al., U. S. Patent No. 4, 468, 469]. Compounds that are related to fenclofenac in their ability to displace thyroid hormones from serum binding proteins have also shown utility in the present invention. In the present invention fenclofenac (Y = O, R^1 = Cl, R^2 = H, J = CH_2CO_2H) is a preferred compound.

It has been discovered that the tracers and antibodies raised against immunogens of the present invention produce excellent results in a fluorescence polarization assay of the present invention for the semi-quantitative detection of PCBs. However, other assays employing the use of antibodies and antigens also can be used.

The assay of the present invention is performed in accordance with the following general procedure. A known volume of standard or extracted test sample containing or suspected of containing PCBs is delivered to a container such as a test tube. A known concentration of tracer is added to the tube. A known concentration of analyte-specific antibody, produced using the immunogen as described above, also is added to the tube. This reaction mixture is incubated under conditions and for a sufficient time, during which time the tracer and analyte compete for limited antibody binding sites, and whereby tracer-antibody and analyte-antibody complexes form. The amount of tracer-antibody complex formed is measured to determine the presence and/or amount of the analyte in the test sample.

The assay is adaptable to be performed on automated systems such as, but not limited to, the TD_x® Therapeutic Drug Monitoring System, the AD_x™ Abused Drug System, the IM_x® Fluorescence Polarization Analyzer and Microparticle Enzyme Immunoassay Analyzer, all of which are available from Abbott Laboratories, Abbott Park, Illinois. When either the TD_x®, AD_x™, or the IM_x® system is used, the assays are fully automated, from pretreatment to final reading once the test sample has been prepared. Manual assays, however, can also be performed. Although the method of the invention are applicable to manual assays, the automated nature of the TD_x®, AD_x™ and the IM_x® systems assures minimal technician time to perform assays and interpret data.

When using fluorescence polarization, the results can be quantified in terms of "millipolarization units", "span" (in millipolarization units) and "relative intensity". The measurement of millipolarization units indicates the maximum polarization when a maximum amount of the tracer is bound to the antibody in the absence of any PCB in the test sample. The higher the net millipolarization units, the better the binding of the tracer to the antibody. For the purposes of the present invention, a net millipolarization value of at least 150 is preferred for a cutoff level of 5µg/mL of aroclor.

The "span" is an indication of the difference between the net millipolarization and the minimum amount of tracer bound to the antibody. A larger span provides for a better numerical analysis of the data. For the purposes of the present invention, a span of at least 15 millipolarization units is preferred.

The "relative intensity" is a measure of the strength of the fluorescence signal above the background fluorescence. Thus, a higher intensity will give a more accurate measurement. The intensity is determined as the sum of the vertically polarized intensity plus twice the horizontally polarized intensity. The intensity can range from a signal of about three times to about thirty times the background noise, depending upon the concentration of the tracer and other assay variables. For the purposes of the present invention, an intensity of about three to about twenty times that of background noise is preferred.

The pH at which the method of the present invention is practiced must be sufficient to allow the fluorescein moiety of the tracers to exist in their open form. The pH can range from about 4 to 9, preferably from about 6 to 8, and most preferably from about 7 to 7.5. Various buffers can be used to achieve and maintain the pH during the assay procedure. Representative buffers include borate, phosphate, carbonate, Tris, barbital and the like. The choice of the particular buffer used is not critical to the practice of the present invention. However, Tris and phosphate buffers are preferred.

The present invention will now be described by way of specific examples, which are meant to illustrate, but not to limit, the scope of the invention.

EXAMPLES

Synthesis of Haptens

Example 1: *2-Amino-2',4,4',5,5'-pentachlorobiphenyl*

A. *2',4,4',5,5',-pentachlorobiphenyl* was prepared according to the standard procedure of Cadogan [J. I. G. Cadogan, *J. Chem. Soc. (London)*, 1962, 4257-58]. Thus, 2,4,5-tricholoroaniline (20g, 0.1mol) was dissolved in 1,2-dichlorobenzene (125mL, 1.1mol). t-Butyl nitrite (28.2mL, 1.1mol) was introduce to the stirred solution in three portions over 10min. After 15min the evolution of gas had subsided, and the

reaction mixture was heated to reflux behind a safety shield for 90min, and then stirred at ambient temperature for 12h. The excess 1,2-dichlorobenzene was removed *in vacuo* and the residue containing the product was purified by filtration through silica gel following the method of E. K. Yau and J. K. Coward, *Aldrichimica Acta*, 1988, *21*(4), 106-107, to give after crystallization from ethanol the title product (3g, 9.3%). Mass spectrum: *m/z* at 324 for $C_{12}H_5Cl_5$.

B. *2-Nitro-2',4,4',5,5'-pentachlorobiphenyl* was prepared according to the standard procedure as described by W. H. Newsome and J. B. Shields, *Intern. J. Environ. Anal. Chem.*,1981, *10*, 295-304]. Thus, 2',4,4',5,5'-pentachlorobiphenyl (2.8g, 8.9mmol) was added to a solution of concentrated nitric and sulfuric acid (50mL, 1:1) at 0°C with stirring. The reaction was allowed to warm to ambient temperature, then was heated to 60°C for 2h. Finally, after stirring for 48h at ambient temperature, the reaction mixture was poured into ice (100g), filtered, and air dried to give the title compound (3g). Mass spectrum: *m/z* at 414 for $C_{12}H_4Cl_5NO_2$.

C. *2-Amino-2',4,4',5,5'-pentachlorobiphenyl* was prepared from 2-Nitro-2',4,4',5,5'-pentachlorobiphenyl (2.5g, 6.8mmol) by dissolving the compound in acetic acid (60mL) heated to 100°C and adding a solution of $SnCl_2$ (6g) in concentrated HCl (30mL). The temperature was raised to reflux for 3h, then the reaction mixture was allowed to cool to ambient temperature, whereupon it was poured into ice (100g). The pH of the solution was adjusted to 9 with NaOH and then extracted with methylene chloride (2x100mL). The extract was washed with NaOH (1N, 2x25mL) and water (100mL); dried over $MgSO_4$ and filtered to give the impure amine. The crude product was chromatographed in two equal batches (Chromatotron®, Harrison Research, Palo Alto, California, 4mm silica gel, step gradient from hexanes to 10% ethyl acetate in hexanes, 20 mL/min) to give the title hapten (830mg). Mass spectrum: *m/z* at 340 (M + 1) for $C_{12}H_6Cl_5N$.

Example 2: *2,4'-Dichloro-4-amino-biphenyl*

A. *2,4'-dichloro-4-nitro-biphenyl* was prepared by dissolving 2-chloro-4-nitroaniline (2.6g, 15mmol) in chlorobenzene (20mL, 200mmol), heated to 120°C under a nitrogen atmosphere. *i*-Amylnitrite (3.4mL, 25mmol) was added to the solution slowly over 1h *via* a syringe pump and canula. After stirring for 12h the reaction mixture was cooled to ambient temperature and the excess chlorobenzene was removed *in vacuo*. The residue after evaporation was chromatographed by filtration through silica gel as in Example 1A, using mixture of hexane and ethyl acetate (80:20) as the eluent. Further purification was achieved by chromatography [Chromatotron®, 4mm silica gel, hexanes/methylene chloride, 80:20, 20 mL/min] which yielded the title compound (1.75g). Mass spectrum: *m/z* at 267 for $C_{12}H_7Cl_2NO_2$.

B. *2,4'-Dichloro-4-amino-biphenyl* was prepared from 2,4'-dichloro-4-nitro-biphenyl (1.75g, 6.5mmol) according to the procedure in Example 1C. The crude material was purified by chromatography [Chromatotron®, 4mm silica gel, hexanes/methylene chloride, 80:20, 20 mL/min] followed by preparative thin layer chromatography [Whatman PLKC18F, 1mm, 20x20cm reverse phase plates, methanol/1% aq. acetic acid, 75:25]. Two major components were isolated as oils. The faster eluting component (480mg) was identified as 2,2'-dichloro-4-amino-biphenyl, while the slower eluting component was the title compound (580mg). Mass spectrum: *m/z* at 238 (M + 1) for $C_{12}H_9Cl_2N$.

Example 3: *2,5,5'-Trichloro-2'-aminobiphenyl*

A. *2,5,5'-Trichloro-2'-nitrobiphenyl* was prepared according to the procedure in Example 2A from 2-nitro-5-chloroaniline (2.6g, 15mmol) and 1,4-dichlorobenzene (29.5g, 200mmol). Purification was by filtration through silica gel (see Example 1A) eluting with hexanes/methylene chloride 80:20 yielding the title compound (830mg). Mass spectrum: *m/z* at 301 for $C_{12}H_6Cl_3NO_2$.

B. *2,5,5'-Trichloro-2'-aminobiphenyl* was prepared from *2,5,5'-trichloro-2'*-nitrobiphenyl (830mg, 2.8mmol) according to the procedure of example 1C. Purification was by chromatography [Chromatotron®, 2mm silica gel, ethyl acetate/hexanes, 50:50, 10 mL/min] and yielded the title compound (448mg). Mass spectrum: *m/z* at 272 (M + 1) for $C_{12}H_8Cl_3N$.

Example 4: *5-Hydroxy-2,2',4,4',5'-pentachlorobiphenyl*

A. *2,4-Dichloroanisole.* 2,4-Dichlorophenol (50g,0.3mol) was dissolved in 2-butanone (500mL) along with K_2CO_3 (200g), then treated with methyl iodide (90mL, 1.45mol) by the dropwise addition over 2h to the stirred solution. The solution was heated to reflux for 12h, cooled, filtered, and evaporated. Distillation (Kugelrohr, 5mm Hg, bT 110°C) gave the title compound (58g). Mass spectrum: *m/z* at 176 for

$C_7H_6Cl_2O$.

B. *5-Methoxy-2,2',4,4',5'-pentachlorobiphenyl* was prepared from 2,4,5-trichloroaniline (2.95g, 15mmol) and 2,4-Dichloroanisole (35g, 200mmol) according to the procedure of Example 2A. Distillation of the crude reaction mixture (Kugelrohr, 1mm Hg, bT 190 ° C) gave a mixture of 5-Methoxy-2,2',4,4',5'-pentachlorobiphenyl and 2-Methoxy-2',4,4',5,5'-pentachlorobiphenyl. The isomers were separated by chromatography [Chromatotron®, 4mm silica gel, heptane, 20 mL/min]. The first eluting isomer was identified as 2-Methoxy-2',4,4',5,5'-pentachlorobiphenyl (383mg) while the second was the desired 5-Methoxy-2,2',4,4',5'-pentachlorobiphenyl (429mg). Mass spectrum: *m/z* at 354 for $C_{13}H_7Cl_5O$.

C. *5-Hydroxy-2,2',4,4',5'-pentachlorobiphenyl* was prepared from 5-Methoxy-2,2',4,4',5'-pentachlorobiphenyl (400mg, 1.2mmol) according to the procedure outlined by J. F. W. McOmbie, et al., *Tetrahedron,* 1967, *24,* 2289-92]. Yield: 260mg. Mass spectrum: *m/z* at 340 for $C_{12}H_5Cl_5O$.

Example 5: *4-Hydroxy-2,2',4',5,5'-pentachlorobiphenyl*

A. *2,5-Dichloroanisole* was prepared from 2,5-dichlorophenol (50g, 0.3mol) according to the procedure in Example 4A. Distillation (bp 98 ° C, 5mm Hg) gave the product (52g). Mass spectrum: *m/z* at 176 for $C_7H_6Cl_2O$.

B. *4-Methoxy-2,2',4',5,5'-pentachlorobiphenyl* was prepared from 2,5-Dichloroanisole (35g, 200mmol) and 2,4,5-trichloroaniline (2.95g, 15mmol) according to the procedure in Example 2A. The excess dichloroanisole was removed by distillation and the residue was then filtered through silica gel (heptane eluent) and further purified [Chromatotron®, 4mm silica gel, hexane, 20 mL/min] to give the product (980mg). Mass spectrum: *m/z* at 354 for $C_{13}H_7Cl_5O$.

C. *4-Hydroxy-2,2',4',5,5'-pentachlorobiphenyl* was prepared from 4-Methoxy-2,2',4',5,5'-pentachlorobiphenyl (950mg, 2.7mmol) according to the procedure in Example 4C. Yield: 918mg. Mass spectrum: *m/z* at 340 for $C_{12}H_5Cl_5O$.

Example 6: *2-Hydroxy-2',4,4',5,5'-pentachlorobiphenyl*

A. *3,4-Dichloroanisole* was prepared from 3,4-dichlorophenol (50g, 0.3mol) according to the procedure in Example 4A. Distillation (bp 75-80 ° C, 1mm Hg) gave the product (56g). Mass spectrum: *m/z* at 176 for $C_7H_6Cl_2O$.

B. *2-Methoxy-2',4,4',5,5'-pentachlorobiphenyl* was prepared from 3,4-Dichloroanisole (35g, 200mmol) and 2,4,5-trichloroaniline (2.95g, 15mmol) according to the procedure in Example 2A. The excess dichloroanisole was removed by distillation and the residue was then filtered through silica gel (heptane eluent) and further purified [Chromatotron®, 4mm silica gel, cyclohexane, 20 mL/min] to give 2-methoxy-2',4,4',5,5'-pentachlorobiphenyl (700mg) and 2-Methoxy-2',4',5,5',6-pentachlorobiphenyl (1.2g). Mass spectrum: *m/z* at 354 for $C_{13}H_7Cl_5O$.

C. *2-Hydroxy-2',4,4',5,5'-pentachlorobiphenyl* was prepared from 42-methoxy-2',4,4',5,5'-pentachlorobiphenyl (700mg, 2mmol) according to the procedure in Example 4C. Yield: 295mg. Mass spectrum: *m/z* at 340 for $C_{12}H_5Cl_5O$.

Example 7: *2-Chloro-4-hydroxybiphenyl*

A. *2-Chloro-4-methoxybiphenyl* was prepared from p-anisidine (1.85g, 15mmol) and chlorobenzene (50mL) according to the procedure of example 2A. The excess chlorobenzene was removed by distillation and the residue was then filtered through silica gel (cyclohexane/ ethyl acetate, 95:5 eluent) to give the product (860mg). Mass spectrum: *m/z* at 236 ($M + NH_4$) for $C_{13}H_{11}ClO$.

B. *2-Chloro-4-hydroxybiphenyl* was prepared from 4 2-Chloro-4 methoxybiphenyl (437mg, 2mmol) according to the procedure in Example 4C. Yield: 408mg. Mass spectrum: *m/z* at 204 for $C_{12}H_9ClO$.

Synthesis of Haptens with Linker arms

Example 8: *2-Adipamido-2',4,4',5,5'-pentachlorobiphenyl*

A. Chloro methyl adipate was prepared from mono-methyl adipate (10g, 62.4mmol) by refluxing with thionyl chloride (20mL) for 2h. Distillation gave the acid chloride (bp 80 ° C,0.35mm Hg, 7.4g).

B. Methyl *2-Adipamido-2',4,4',5,5'-pentachlorobiphenyl* was prepared from 2-Amino-2',4,4',5,5'-pentachlorobiphenyl (300mg, 0.88mmol) by treatment with chloro methyl adipate (180mg, 1mmol) in pyridine

(5mL) for 12h at ambient temperature. The mixture was then added to ethyl ether (50mL) and washed with aq HCl (1.2N, 4x25mL), saturated $NaHCO_3$ (2x20mL), brine (2x20mL) and dried over $MgSO_4$ and filtered. Evaporation gave the product (440mg). Mass spectrum: *m/z* at 482 (M + 1) for $C_{19}H_{16}Cl_5NO_3$.

C. *2-Adipamido-2',4,4',5,5'-pentachlorobiphenyl* was prepared by saponification (ethanolic NaOH, reflux, 2h) to the ester in example 8B. The product was isolated by the addition of ethyl ether (100mL); washing with aq. HCl (1.2N, 15mL), brine (20mL); drying over $MgSO_4$, filtering and evaporating. Yield: 226mg. Mass spectrum: *m/z* at 468 (M + 1) for $C_{18}H_{14}Cl_5NO_3$.

Example 9: *2,4'-Dichloro-4-adipamidobiphenyl*

A. Methyl *2,4'-dichloro-4-adipamidobiphenyl* was prepared from 2,4'-dichloro-4-amino-biphenyl (300mg, 1.26mmol) according to the procedure in example 8B. Yield: 550mg. Mass spectrum: *m/z* at 380 (M + 1) for $C_{19}H_{19}Cl_2NO_3$.

B. *2,4'-Dichloro-4-adipamidobiphenyl* was prepared by saponification (ethanolic NaOH, reflux, 2h) to the ester as in example 8B. The product was isolated by adding ethyl ether (100mL); washing with aq. HCl (1.2N, 15mL), brine (20mL); drying over $MgSO_4$, filtering and evaporating. Yield: 233mg. Mass spectrum: *m/z* at 366 (M + 1) for $C_{18}H_{17}Cl_2NO_3$.

Example 10: *2,5,5'-Trichloro-2'-adipamidophenyl*

A. *Methyl 2,5,5'-trichloro-2'-adipamidobiphenyl* was prepared from 2,5,5'-trichloro-2-aminobiphenyl (300mg,1.1mmol) according to the procedure in example 8B. Yield: 44Omg. Mass spectrum: *m/z* at 414 (M + 1) for $C_{19}H_{18}Cl_3NO_3$.

B. *2,5,5'-Trichloro-2'-adipamidobiphenyl* was prepared by saponification (ethanolic NaOH, reflux, 2h) to the ester as in example 8B. The product was isolated by the addition of ethyl ether (100mL); washing with aq. HCl (1.2N, 15mL), brine (20mL); drying over $MgSO_4$, filtering and evaporating. Yield: 260mg. Mass spectrum: *m/z* at 400 (M + 1) for $C_{18}H_{16}Cl_3NO_3$.

Example 11: *5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl*

A. *Ethyl 5-(methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl* wasprepared from 5-hydroxy-2,2',4,4',5'-pentachlorobiphenyl (200mg, 0.58mmol, Example 4C) and ethyl bromoacetate (80μL, 0.73mmol) by dissolving both in 2-butanone (10mL) in the presence of K_2CO_3 (100mg, 0.725mmol) and a catalytic amount of NaI. The reaction mixture was stirred under a nitrogen atmosphere at reflux for 16h; cooled; diluted with ethyl ether; washed with H_3PO_4 (1.4M, 50mL) and brine (25mL); dried over $MgSO_4$; and evaporated to give the product. Mass spectrum: *m/z* at 446 (M + NH_4) for $C_{16}H_{11}Cl_5O_3$.

B. *5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl* was prepared by saponification of ethyl 5-(methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenylusing methanolic KOH (20mL, 200mg) at ambient temperature for 5h. The solution was then evaporated and the residue suspended in H_3PO_4 (1.4M, 25mL); extracted with ethyl ether (4x25mL); the extract was washed with brine (25mL); dried over $MgSO_4$; and evaporated to give the product. Mass spectrum: *m/z* at 416 (M + NH_4) for $C_{14}H_7Cl_5O_3$.

Example 12: *4-(Methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl*

A. *Ethyl 4-(methoxycarboxylato)-2,2',4',5,5',-pentachlorobiphenyl* was prepared from 4-hydroxy-2,2',4',5,5'-pentachlorobiphenyl (650mg, 1.9mmol, Example 5C) by the method in example 11A. Purification by chromatography [Chromatotron®, 2mm silica gel, 5% ethyl acetate in cyclohexane, 10 mL/min] yielded the title compound (715mg). Mass spectrum: *m/z* at 446 (M + NH_4) for $C_{16}H_{11}Cl_5O_3$.

B. *4-(Methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl* was prepared from Ethyl 4-(methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl (700mg, 1.65mmol) by the method of example 11B (520mg). Mass spectrum: *m/z* at 416 (M + NH_4) for $C_{14}H_7Cl_5O_3$.

Example 13: *2-(Methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl*

A. *Ethyl 2(methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl* was prepared from 2-hydroxy-2',4,4',5,5'-pentachlorobiphenyl (295mg, 0.86mmol, Example 6C) by the method in example 11A (438mg). Mass spectrum: *m/z* at 446 (M + NH_4) for $C_{16}H_{11}Cl_5O_3$.

B. *2-(Methoxycarboxylato)-2',4,4',5,5',-pentachlorobiphenyl* was prepared from Ethyl 2-

(methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl (438mg) by the method of example 11B (200mg). Mass spectrum: *m/z* at 416 ($M + NH_4$) for $C_{14}H_7Cl_5O_3$.

Example 14: *4-(Butoxy-4-carboxylato)-2-chlorobiphenyl*

A. *Ethyl 4-(butoxy-4-carboxylato)-2-chlorobiphenyl* was prepared from 2-chloro-4-hydroxybiphenyl (408mg, 2.0mmol, Example 6C) and ethyl 4-bromobutyrate (290mL, 2.05mmol) by the method in example 11A. Purification by chromatography [Chromatotron®, 4mm silica gel, 10% ethyl acetate in cyclohexane, 20 mL/min] yielded the title compound (585mg). Mass spectrum: *m/z* at 319 ($M + 1$), 336 ($M + NH_4$) for $C_{18}H_{19}Cl_1O_3$.

B. *4-(Butoxy-4-carboxylato)-2-chlorobiphenyl* was prepared from *ethyl 4-(butoxy-4-carboxylato)-2-chlorobiphenyl* (585mg, 1.8mmol)) by the method of example 11B (462mg). Mass spectrum: *m/z* at 308 ($M + NH_4$) for $C_{16}H_{15}Cl_1O_3$.

Synthesis of Immunogens

Example 15

A. General procedure I: The hapten (25mg) was activatated with dicyclohexylcarbodiimide (DCC, 15mg, 0.07mmol) and N-hydroxysuccinimide (NHS, 25mg, 0.2mmol) in tetrahydrofuran (5mL, freshly distilled from benzophenone ketyl) at 0 °C for 2h and at ambient temperature for 12h under a nitrogen atmosphere. Bovine serum albumin (BSA, 200mg) was dissolved in phosphate buffer (10mL, 0.1M, pH 8.0). The solution of the activated hapten was filtered through a plug of glass wool into the stirred solution of the BSA. Stirring was continued for 24h, after which the reaction mixture was transferred to dialysis tubing (molecular weight cutoff: 15,000) and dialysed against ammonium formate (6L, 0.1N) at 4 °C for 48h. The dialysate was lyophilized to give a solid which was then washed with chloroform (25mL) and dried in vacuo over P_2O_5. By UV the immunogen contained 13-26 moles of hapten per mole of BSA.

B. General procedure II: The hapten (25mg) was dissolved in thionyl chloride (1mL) and heated to 60 °C for 12h. Afterwards the excess thionyl chloride was removed in vacuo, leaving the acid chloride of the hapten. The acid chloride was then dissolved in THF (2mL, freshly distilled from benzophenone ketyl). BSA (200mg) was dissolved in phosphate buffer (10mL, 0.1M, pH 8.0) and cooled to 0 °C. The solution of the acid chloride was added at once with stirring, along with more THF (8mL). Stirring was continued for 2h at ambient temperature, afterwhich the reaction mixture was transferred to dialysis tubing (molecular weight cutoff: 15,000) and dialysed against ammonium formate (6L, 0.1N) at 4 °C for 48h. The dialysate was lyophilized to give a solid which was then washed with chloroform (25mL) and dried in vacuo over P_2O_5. By UV the immunogen contained 13-26 moles of hapten per mole of BSA.

Example 16

(2-Adipamido-2',4,4',5,5'-pentachlorobiphenyl)$_x$BSA was prepared from 2-Adipamido-2',4,4',5,5'-pentachlorobiphenyl (example 8C) by the procedure in example 15A.

Example 17

(2,4'-dichloro-4-adipamidobiphenyl)$_x$BSA was prepared from 2,4'-dichloro-4-adipamidobiphenyl (Example 9B) by the procedure in example 15A.

Example 18

*(2,5,5'-Trichloro-2'-adipamidobiphenyl)$_x$BSA*was prepared from 2,5,5'-trichloro-2'-adipamidobiphenyl (Example 10B) by the procedure in example 15A.

Example 19

[5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl]$_x$BSA was prepared from 5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl (Example 11B) by the procedure in example 15B.

Example 20

[4-(methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl]$_x$BSA was prepared from 4-(methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl (Example 12B) by the procedure in example 15B.

Example 21

[2-(methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl]$_x$BSA was prepared from 2-(methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl (Example 13B) by the procedure in example 15B.

Example 22

[4-(Butoxy-4-carboxylato)-2-chlorobiphenyl]$_x$BSA was prepared from 4-(Butoxy-4-carboxylato)-2-chlorobiphenyl (Example 14B) by the procedure in example 15A.

Synthesis of Tracers

Example 23

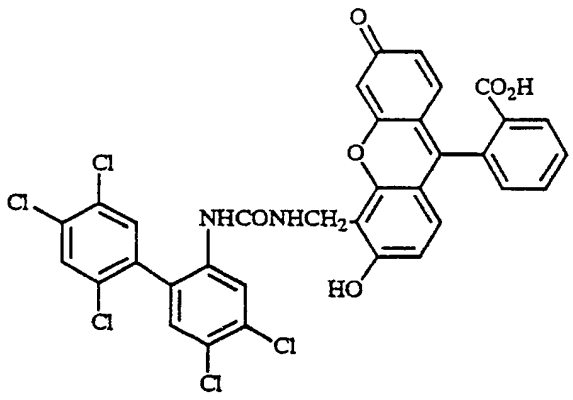
A. General procedure I: The hapten (25mg) was activatated with dicyclohexylcarbodiimide (DCC, 15mg, 0.07mmol) and N-hydroxysuccinimide (NHS, 25mg, 0.2mmol) in tetrahydrofuran (5mL, freshly distilled from benzophenone ketyl) or dimethyl formamide (5mL) at 0 °C for 2h and at ambient temperature for 12h under a nitrogen atmosphere. To an aliquot (1mL) of the activated hapten was added an amino bearing fluorescein derivative (4mg) along with 2 drops of triethylamine. The reaction mixture was stirred for 12h, evaporated and chromatographed [Whatman PLKC18F, 1mm, 20x20cm reverse phase plates, methanol/1% aq. acetic acid, 60:40 or MERCK Silica Gel 60 F-254, 2mm, 20x20cm , chloroform/methanol, 85:15].

B. General procedure II: The hapten (25mg) was dissolved in thionyl chloride (1mL) and heated to 60 °C for 12h. Afterwards the excess thionyl chloride was removed in vacuo, leaving the acid chloride of the hapten. The acid chloride was then dissolved in THF (5mL, freshly distilled from benzophenone ketyl). To an aliquot (1mL) of the activated hapten was added an amino bearing fluorescein derivative (4mg) along with 2 drops of triethylamine. The reaction mixture was stirred for 12h, evaporated and chromatographed [Whatman PLKC18F, 1mm, 20x20cm reverse phase plates, methanol/1% aq. acetic acid, 60:40 or MERCK Silica Gel 60 F-254, 2mm, 20x20cm , chloroform/methanol, 85:15].

C. General procedure III: The amino bearing hapten was converted to its hydrochloride by treatment with ethereal hydrogen chloride. The hapten hydrochloride (50mg) was dissolved in THF (10mL, freshly distilled from benzophenone ketyl) and treated with trichloromethyl chloroformate (100µL) for 30min under a nitrogen atmosphere. Afterwards the volatiles were removed in vacuo and the residue was taken up in DMF, divided into aliquots and treated with an amino bearing fluorescein derivative along with one drop of triethylamine. After stirring for 12h, the reaction mixture was evaporated and chromatographed [Whatman PLKC18F, 1mm, 20x20cm reverse phase plates, methanol/1% aq. acetic acid, 60:40 or MERCK Silica Gel 60 F-254, 2mm, 20x20cm , chloroform/methanol, 85:15].

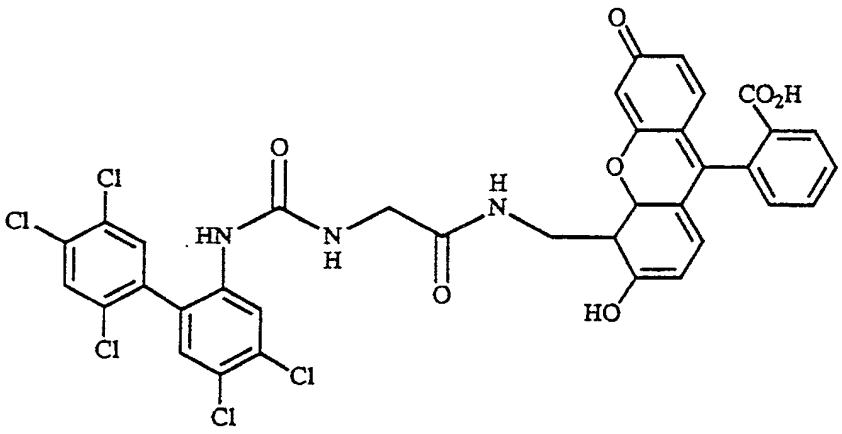
Example 24

A tracer with the following structure was prepared from 2-Amino-2',4,4',5,5'-pentachlorobiphenyl (Example 1C) and 4'-aminomethyl fluorescein according to the method in Example 23C. Mass spectrum (FAB): *m/z* at 727 (M + 1) for $C_{34}H_{19}Cl_5N_2O_6$.



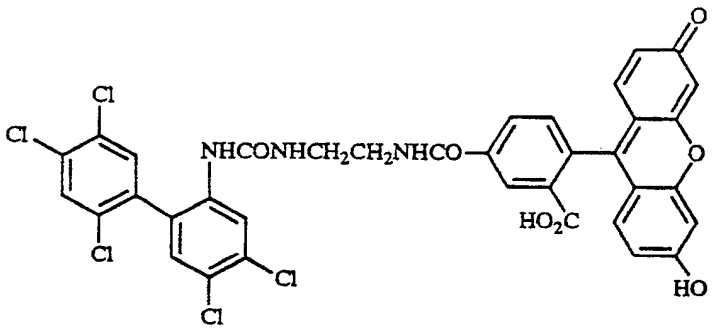
Example 25

A tracer with the following structure was prepared from 2-Amino-2',4,4',5,5'-pentachlorobiphenyl (Example 1C) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23C. Mass spectrum (FAB): *m/z* at 784 (M + 1) for $C_{36}H_{22}Cl_5N_3O_7$.



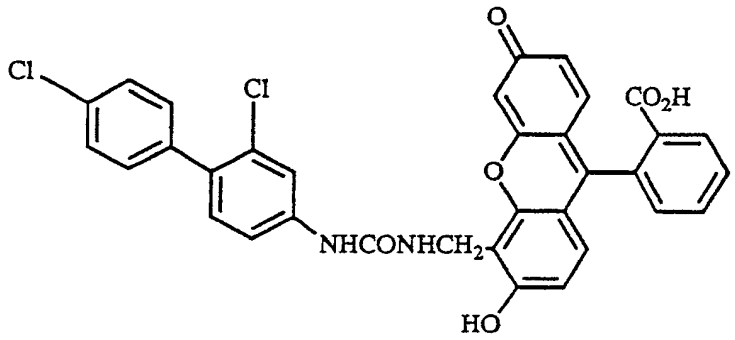
Example 26

A tracer with the following structure was prepared from 2-Amino-2',4,4',5,5'-pentachlorobiphenyl (Example 1C) and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23C. Mass spectrum (FAB): *m/z* at 784 (M + 1) for $C_{36}H_{22}Cl_5N_3O_7$.



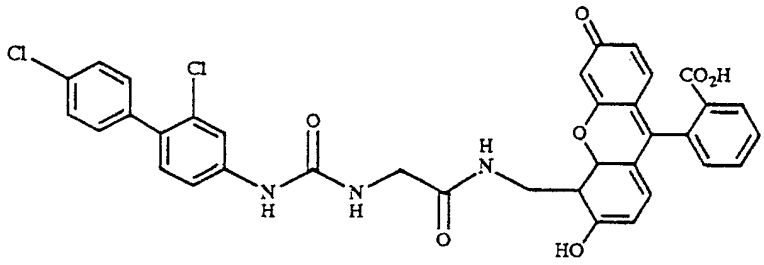
Example 27

A tracer with the following structure was prepared from 2,4'-dichloro-4-amino-biphenyl (Example 2B) and 4'-aminomethyl fluorescein according to the method in Example 23C. Mass spectrum (FAB): *m/z* at 625 (M + 1) for $C_{34}H_{22}Cl_2N_2O_6$.



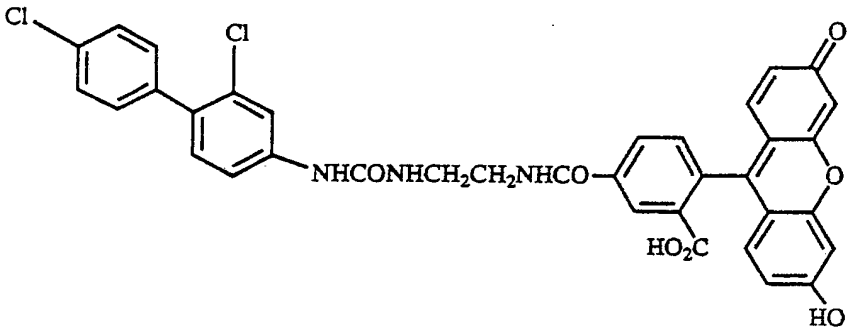
Example 28

A tracer with the following structure was prepared from 2,4'-dichloro-4-amino-biphenyl (Example 2B) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23C. Mass spectrum (FAB): *m/z* at 682 (M + 1) for $C_{36}H_{25}CL_2N_3O_7$.



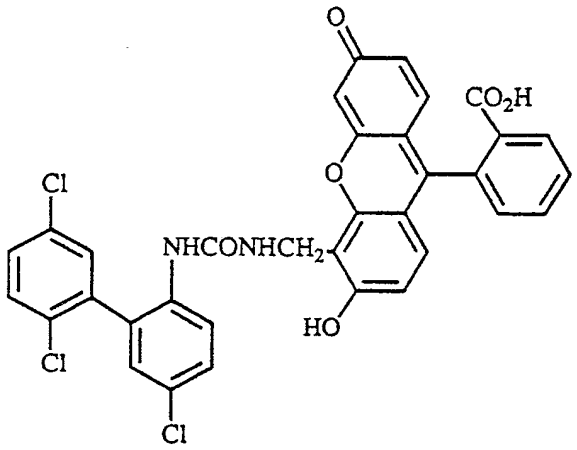
Example 29

A tracer with the following structure was prepared from 2,4'-dichloro-4-amino-biphenyl (Example 2B) and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23C. Mass spectrum (FAB): *m/z* at 682 (M + 1) for $C_{36}H_{25}Cl_2N_3O_7$.



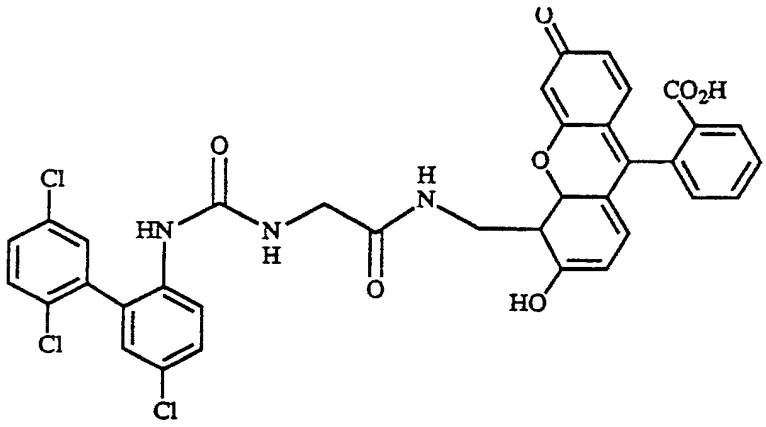
Example 30

A tracer with the following structure was prepared from 2,5,5'-trichloro-2'-aminobiphenyl (Example 3B) and 4'-aminomethyl fluorescein according to the method in Example 23C. Mass spectrum (FAB): *m/z* at 681 (M + Na) for $C_{34}H_{21}Cl_3N_2O_6$.



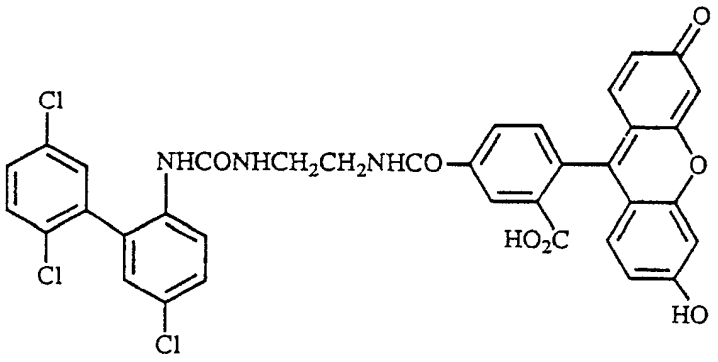
Example 31

A tracer with the following structure was prepared from *2,5,5'-trichloro-2'*-aminobiphenyl (Example 3B) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23C. Mass spectrum (FAB): *m/z* at 716 (M + 1) for $C_{36}H_{24}Cl_3N_3O_7$.



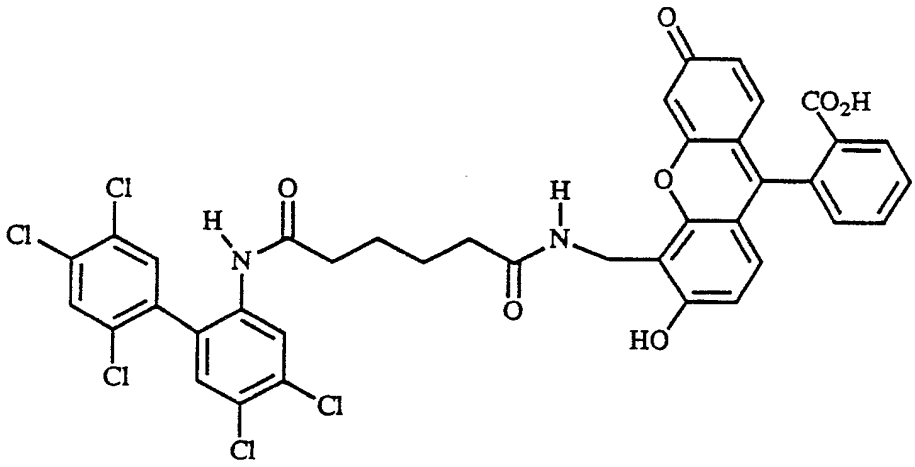
Example 32

A tracer with the following structure was prepared from 2,5,5'-trichloro-2'-aminobiphenyl (Example 3B) (Example 2B) and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23C. Mass spectrum (FAB): *m/z* at 716 (M + 1) for $C_{36}H_{24}Cl_3N_3O_7$.



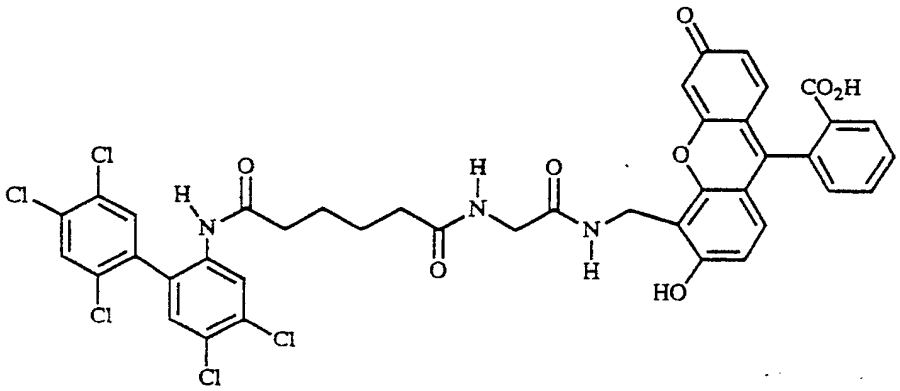
Example 33

A tracer with the following structure was prepared from 2-Adipamido-2',4,4',5,5'-pentachlorobiphenyl (Example 8C) and 4'-aminomethyl fluorescein according to the method in Example 23A.



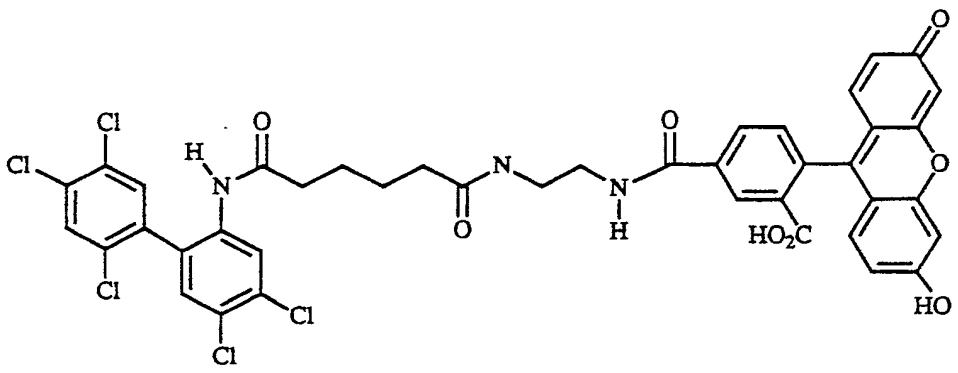
Example 34

A tracer with the following structure was prepared from 2-Adipamido-2',4,4',5,5'-pentachlorobiphenyl (Example 8C) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23A.



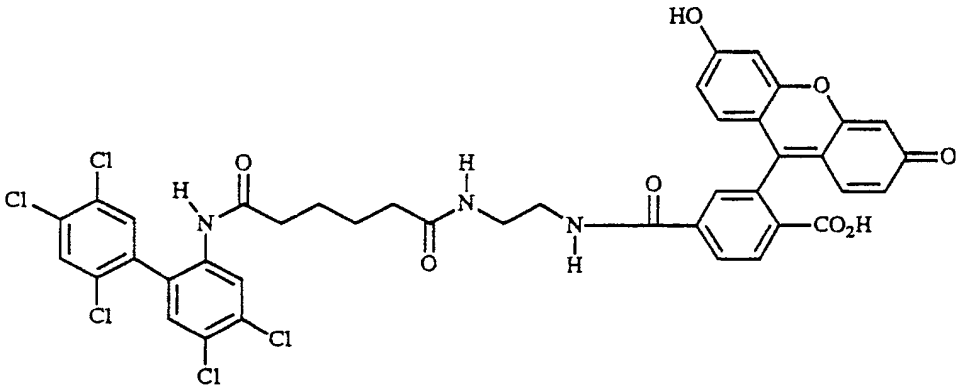
Example 35

A tracer with the following structure was prepared from-Adipamido-2',4,4',5,5'-pentachlorobiphenyl (Example 8C) and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23A.



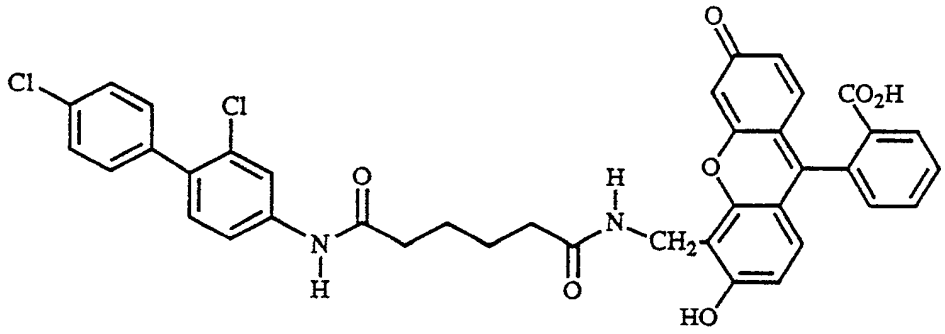
Example 36

A tracer with the following structure was prepared from-Adipamido-2',4,4',5,5'-pentachlorobiphenyl (Example 8C) and 6-[N-(2-aminoethyl)carboxamido]-fluorescein. according to the method in Example 23A.



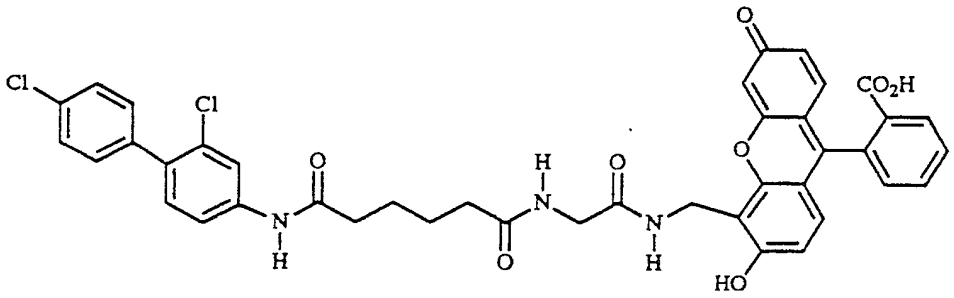
Example 37

A tracer with the following structure was prepared from 2,4'-dichloro-4-adipamidobiphenyl (Example 9B) and 4'-aminomethyl fluorescein according to the method in Example 23A. Mass spectrum (FAB): *m/z* at 709 (M + 1) for $C_{39}H_{30}Cl_2N_2O_7$.



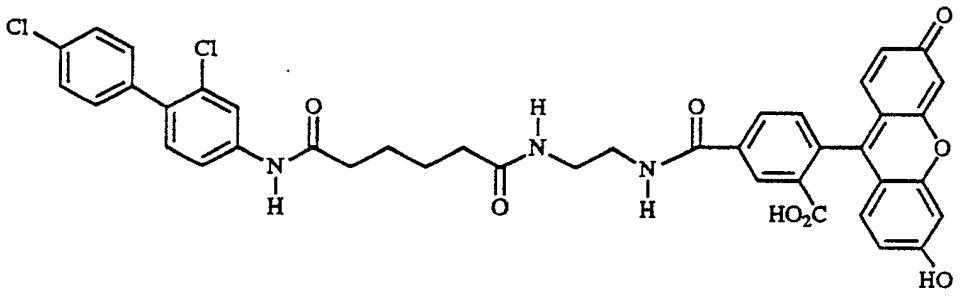
Example 38

A tracer with the following structure was prepared from 2,4'-dichloro-4-adipamidobiphenyl (Example 9B) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23A. Mass spectrum (FAB): *m/z* at 766 (M + 1) for $C_{41}H_{33}Cl_2N_3O_8$.



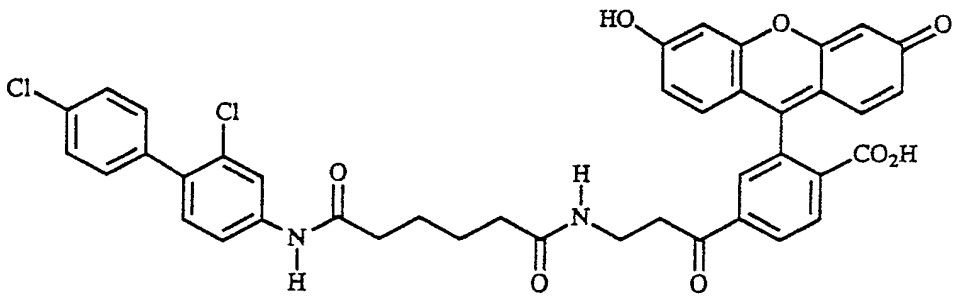
Example 39

A tracer with the following structure was prepared from 2,4'-dichloro-4-adipamidobiphenyl (Example 9B) and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23A. Mass spectrum (FAB): *m/z* at 766 (M + 1) for $C_{41}H_{33}Cl_2N_3O_8$.



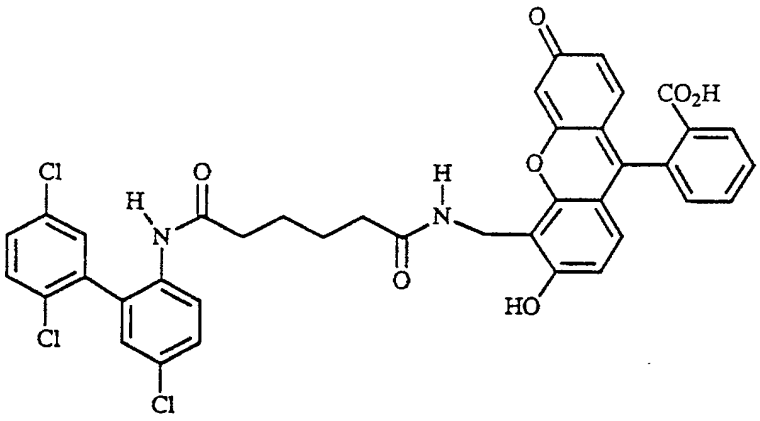
Example 40

A tracer with the following structure was prepared from 2,4'-dichloro-4-adipamidobiphenyl (Example 9B) and 6-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23A. Mass spectrum (FAB): *m/z* at 766 (M + 1) for $C_{41}H_{33}Cl_2N_3O_8$.



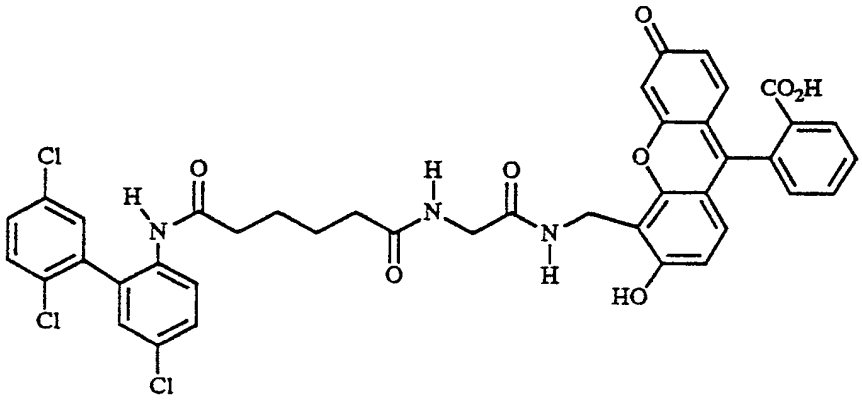
Example 41

A tracer with the following structure was prepared from 2,5,5'-trichloro-2'-adipamidobiphenyl (Example 10B) and 4'-aminomethyl fluorescein according to the method in Example 23A. Mass spectrum (FAB): *m/z* at 743 (M + 1) for $C_{39}H_{29}Cl_3N_2O_7$.



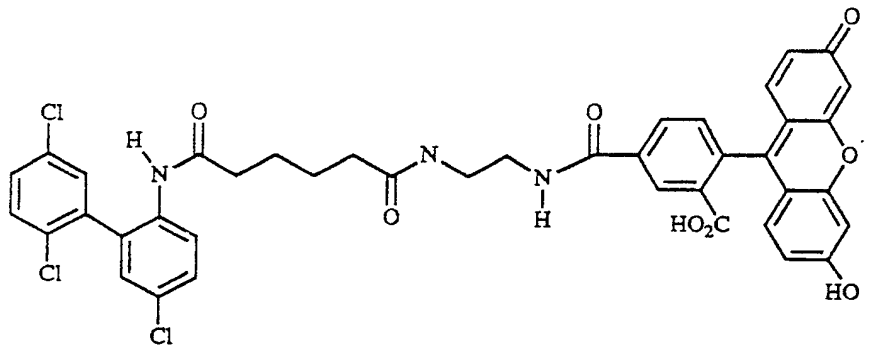
Example 42

A tracer with the following structure was prepared from 2,5,5'-trichloro-2'-adipamidobiphenyl (Example 10B) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23A. Mass spectrum (FAB): *m/z* at 801 (M + 1) for $C_{41}H_{33}Cl_3N_3O_8$.



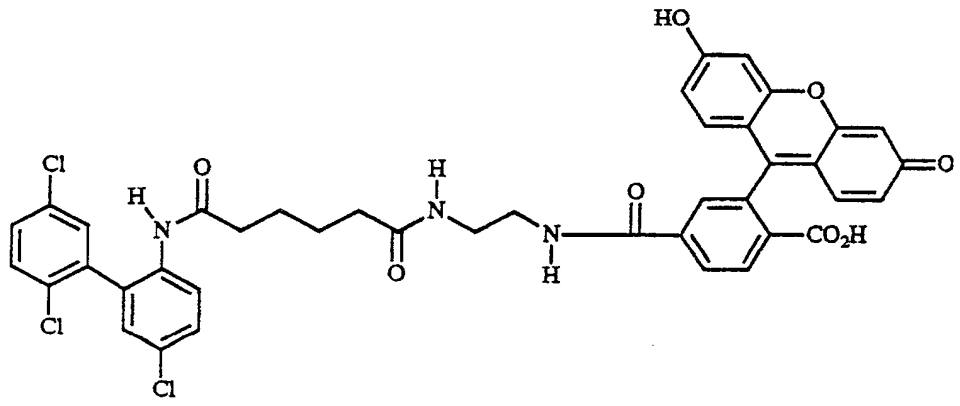
Example 43

A tracer with the following structure was prepared from 2,5,5'-trichloro-2'-adipamidobiphenyl (Example 10B) and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23A. Mass spectrum (FAB): *m/z* at 801 (M + 1) for $C_{41}H_{33}Cl_3N_3O_8$.



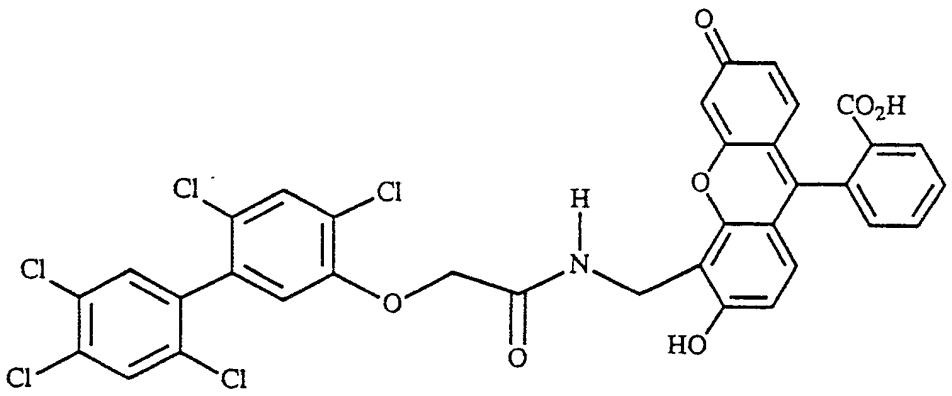
Example 44

A tracer with the following structure was prepared from 2,5,5'-trichloro-2'-adipamidobiphenyl (Example 10B) and 6-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23A. Mass spectrum (FAB): *m/z* at 801 (M + 1) for $C_{41}H_{33}Cl_3N_3O_8$.



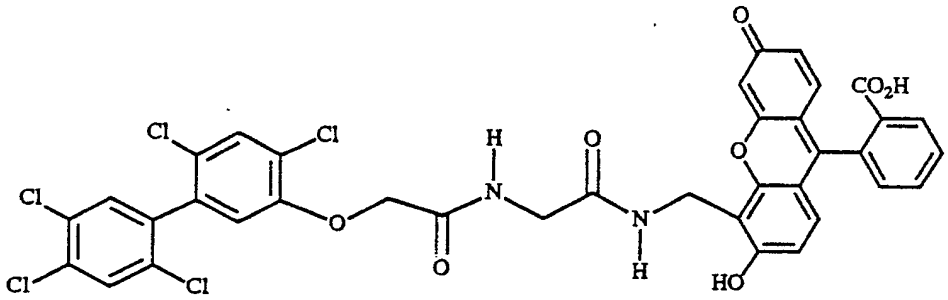
Example 45

A tracer with the following structure was prepared from 5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl (Example 11B) and 4'-aminomethyl fluorescein according to the method in Example 23B.



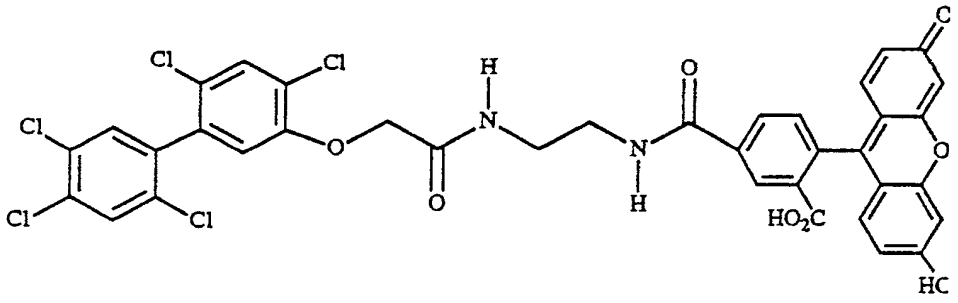
Example 46

A tracer with the following structure was prepared from 5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl (Example 11B) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23B.



Example 47

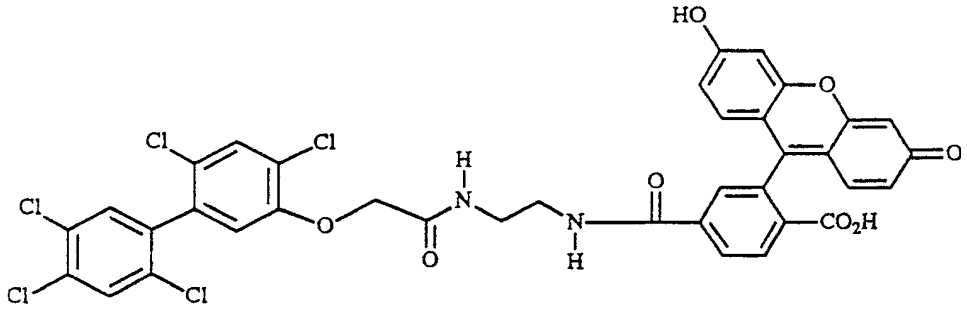
A tracer with the following structure was prepared from 5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl (Example 11B) and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23B.



Example 48

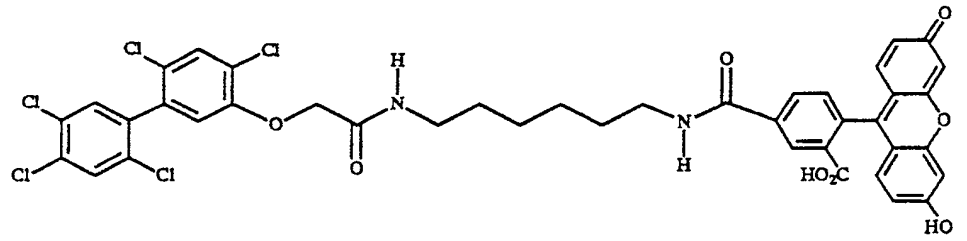
A tracer with the following structure was prepared from 5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl (Example 11B) and 6-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in

Example 23B.



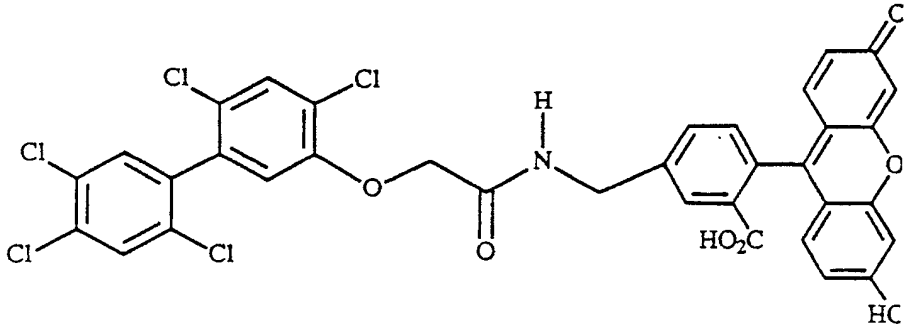
Example 49

A tracer with the following structure was prepared from 5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl (Example 11B) and 5-[N-(6-aminohexyl)carboxamido]-fluorescein according to the method in Example 23B.



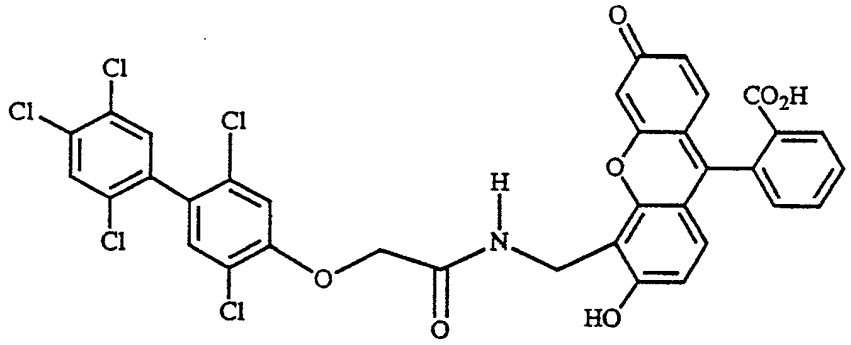
Example 50

A tracer with the following structure was prepared from 5-(Methoxycarboxylato)-2,2',4,4',5'-pentachlorobiphenyl (Example 11B) and 5-aminomethyl fluorescein according to the method in Example 23B.



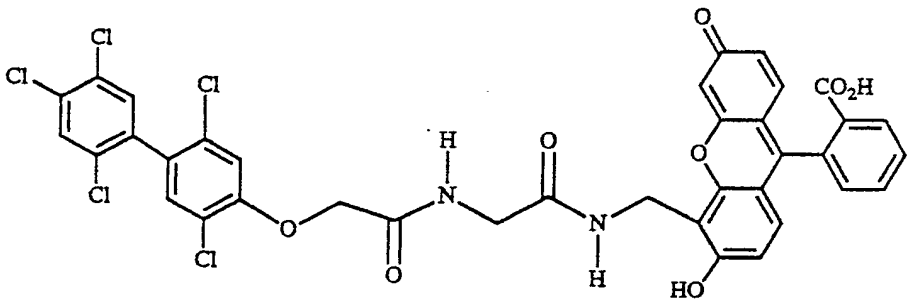
Example 51

A tracer with the following structure was prepared from 4-(methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl (Example 12B) and 4'-aminomethyl fluorescein according to the method in Example 23B.



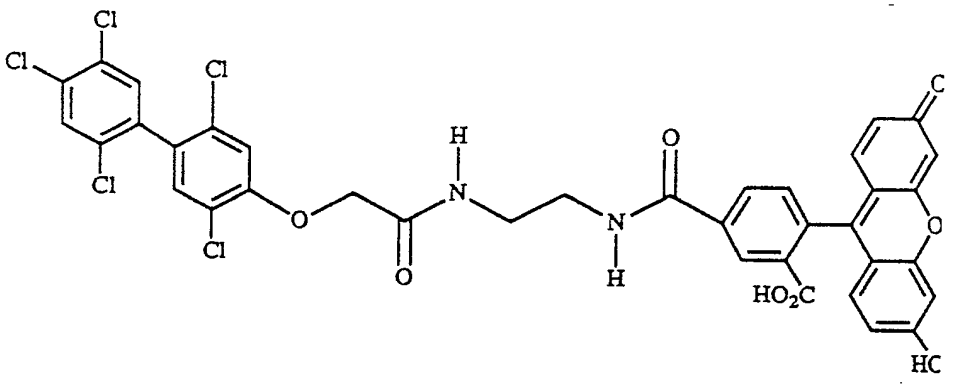
Example 52

A tracer with the following structure was prepared from 4-(methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl (Example 12B) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23B.



Example 53

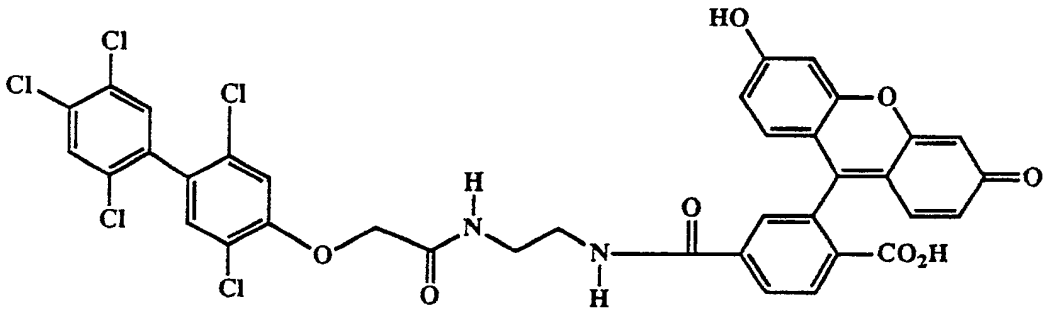
A tracer with the following structure was prepared from 4-(methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl (Example 12B)and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23B.



Example 54

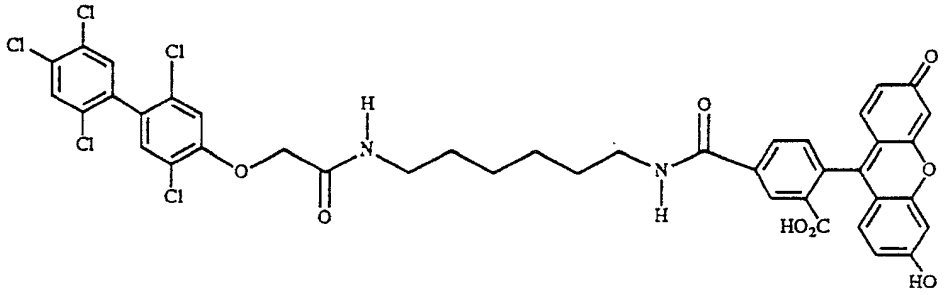
A tracer with the following structure was prepared from 4-(methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl (Example 12B) and 6-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in

Example 23B.



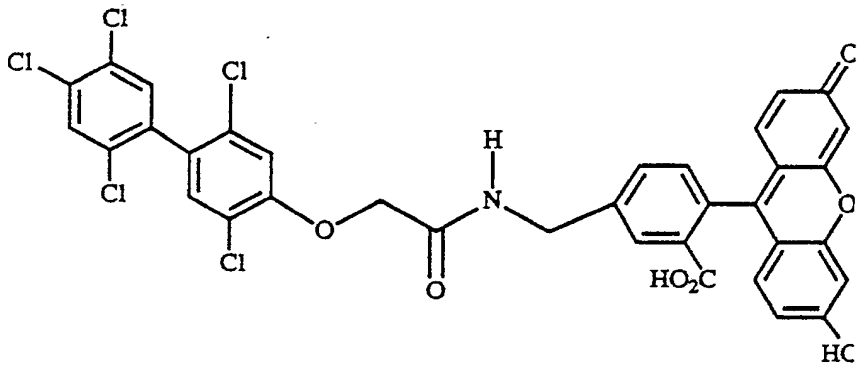
Example 55

A tracer with the following structure was prepared from 4-(methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl (Example 12B) and 5-[N-(6-aminohexyl)carboxamido]-fluorescein according to the method in Example 23B.



Example 56

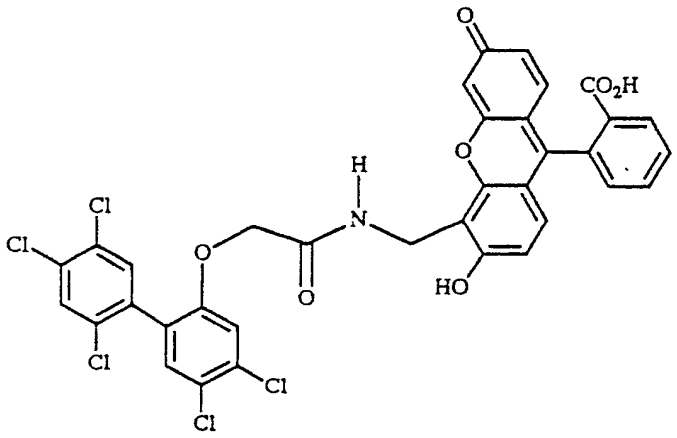
A tracer with the following structure was prepared from 4-(methoxycarboxylato)-2,2',4',5,5'-pentachlorobiphenyl (Example 12B) and 5-aminomethyl fluorescein according to the method in Example 23B.



Example 57

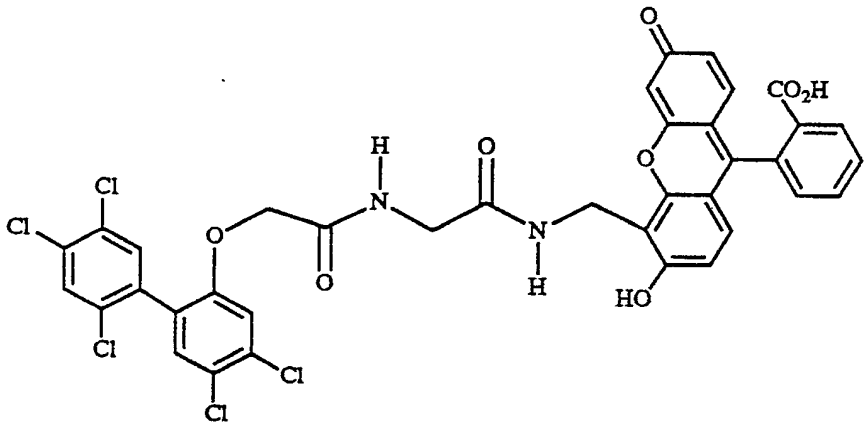
A tracer with the following structure was prepared from 2-(methoxycarboxylato)-2',4,4',5,5'-pentach-

lorobiphenyl (Example 13B) and 4'-aminomethyl fluorescein according to the method in Example 23B.



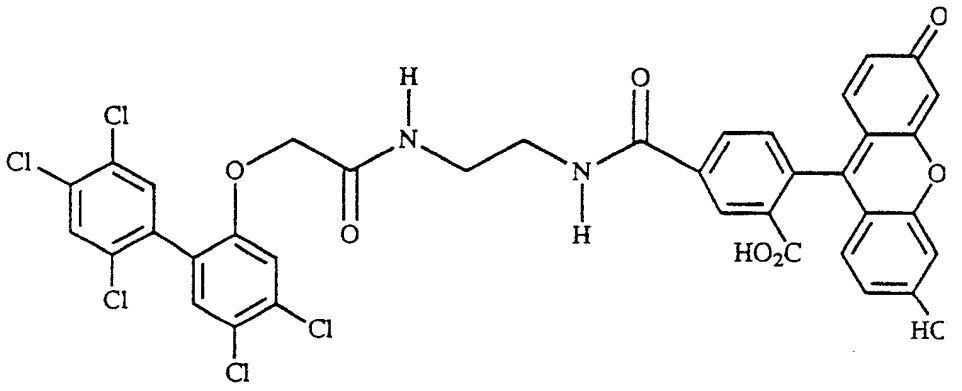
Example 58

A tracer with the following structure was prepared from 2-(methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl (Example 13B) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23B.



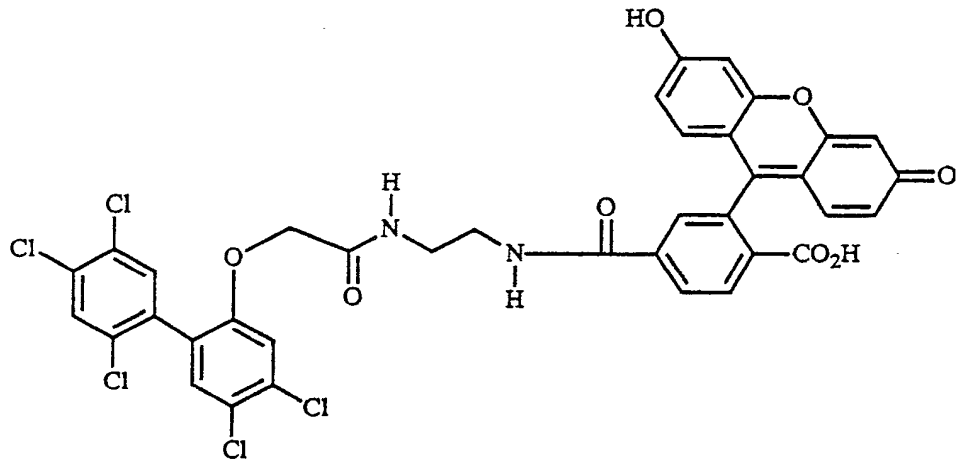
Example 59

A tracer with the following structure was prepared from 2-(methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl (Example 13B) and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23B.



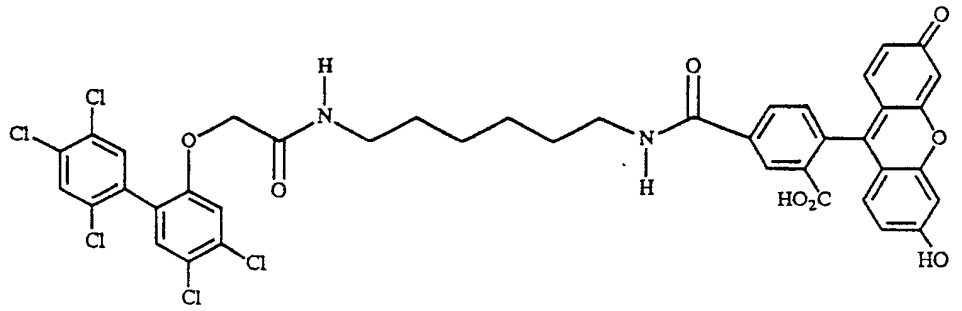
Example 60

A tracer with the following structure was prepared from 2-(methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl (Example 13B) and 6-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23B.



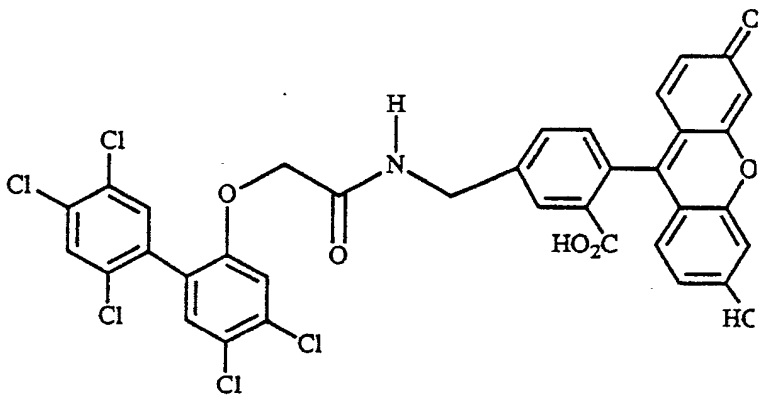
Example 61

A tracer with the following structure was prepared from 2-(methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl (Example 13B) and 5-[N-(6-aminohexyl)carboxamido]-fluorescein according to the method in Example 23B.



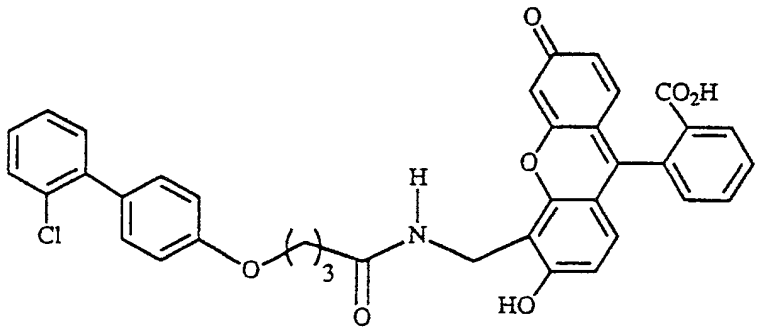
Example 62

A tracer with the following structure was prepared from 2-(methoxycarboxylato)-2',4,4',5,5'-pentachlorobiphenyl (Example 13B) and 5-aminomethyl fluorescein according to the method in Example 23B.



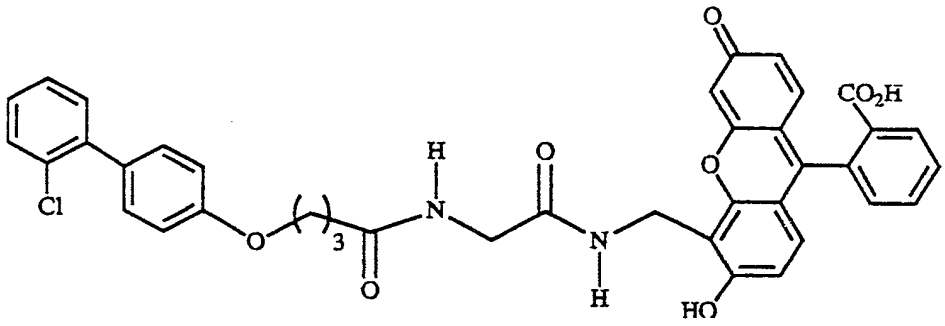
Example 63

A tracer with the following structure was prepared from 4-(Butoxy-4-carboxylato)-2-chlorobiphenyl (Example 14B) and 4'-aminomethyl fluorescein according to the method in Example 23A.



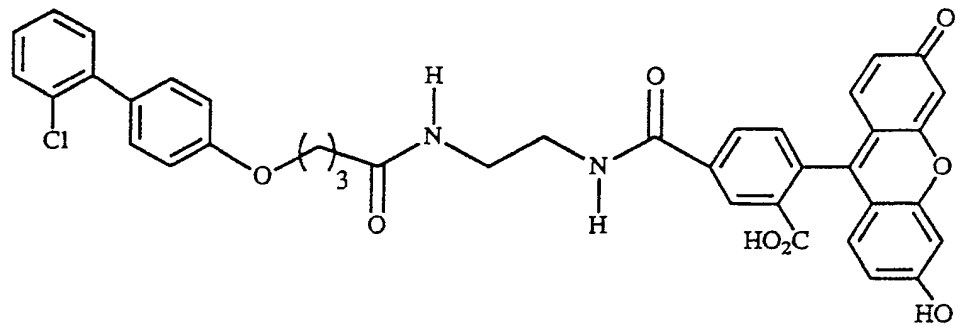
Example 64

A tracer with the following structure was prepared from 4-(Butoxy-4-carboxylato)-2-chlorobiphenyl (Example 14B) and 4'-(N-glycylaminomethyl)-fluorescein according to the method in Example 23A.



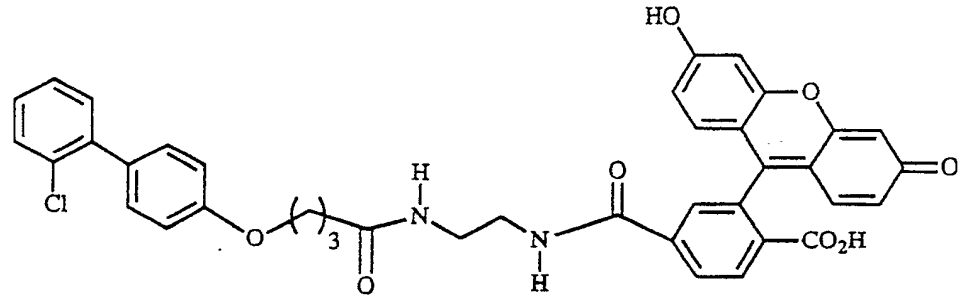
Example 65

A tracer with the following structure was prepared from 4-(Butoxy-4-carboxylato)-2-chlorobiphenyl (Example 14B) and 5-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23A.



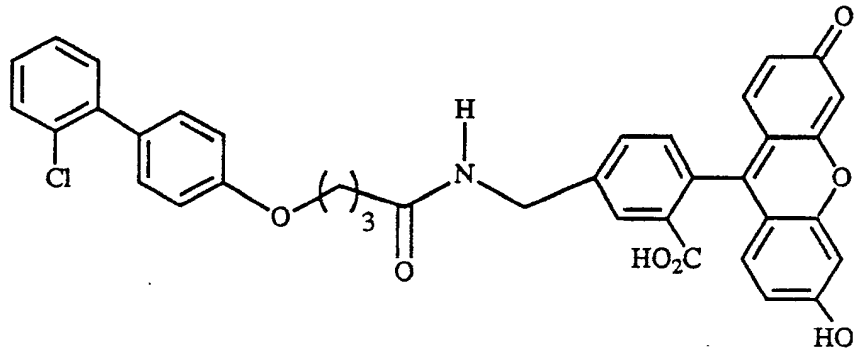
Example 66

A tracer with the following structure was prepared from 4-(Butoxy-4-carboxylato)-2-chlorobiphenyl (Example 14B) and 6-[N-(2-aminoethyl)carboxamido]-fluorescein according to the method in Example 23A.



Example 67

A tracer with the following structure was prepared from 4-(Butoxy-4-carboxylato)-2-chlorobiphenyl (Example 14B) and 5-aminomethyl fluorescein according to the method in Example 23A.



Production of Antisera

Example 68

Female New Zealand White (NZW) rabbits, approximately four to five months old, were injected subcutaneously and intramuscularly with an initial inoculation of 0.2 mg of the immunogen (Examples 16-22) in Freund's Complete Adjuvant followed by a day 14 boost of 0.1 mg of the immunogen, and thereafter monthly booster injections of 0.05 mg in Freund's Incomplete Adjuvant. Bleeds were taken two weeks following each booster injection and the serum tested for binding to tracers and displacement of the tracers from the antibody by PCBs in the TD_x instrument. Antibodies with adequate net millipolarization and span were demonstrated in some bleeds at 6 weeks from initial inoculation.

Production of Hybridomas

Four to six week old female BALB/c mice were injected subcutaneously at four weeks intervals with 0.2 mL of a mixture of each immunogen, (EXAMPLE 16 and EXAMPLE 17), suspended in mixture consisting of: 0.06 mL of immunogen, EXAMPLE 16, at 5mg/mL; 0.06 mL of immunogen, EXAMPLE 17, at 5mg/mL;.1.88 mL saline; and 100µg of monophosphoryl lipid A and trehalose dimycloate adjuvant (Ribi Immunochem Research, Inc). Three months from initial inoculation, upon testing positive for antibody activity on the TD_x instrument,the donor mice were killed by cervical dislocation three days following the last immunization; the spleen was removed aseptically and placed in a plastic Petri dish with 5 mL of cold Dulbecco's Minimal Essential Medium (DMEM),with 2.0 mM L-glutamine and 50 µg/mL Gentamycin (Medium A). The spleen was dissociated into a single cell suspension; the cells were centrifuged to a pellet and the red cells lysed by resuspension in 2 mL of 0.83% ammonium chloride in 10 mM Tris buffer. After letting stand for 2 min., 20-30 mL of fresh medium A was added. The cells were washed by centrifugation and resuspended in 10 mL of fresh medium A.

An immunoglobulin non-secreting mouse myeloma cell line (SP 2/0) deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT-, EC2.4.2.8), as disclosed by Kearney, *Journal of Immunology*, 1979,*123*,1548, which is incorporated herein by reference, was used as the fusion partner. The myeloma cell line was maintained in medium A with 20% fetal calf serum added. For three days prior to fusion, 0.1 mM 8-azaguanine was added to the myeloma cells in order to kill any HGPRT+ revertants. On the day of fusion, the myeloma cells were harvested, washed once in medium A, and resuspended in 5 mL medium A. The myeloma and previously harvested spleen cells were counted using a hemacytometer and their viability assessed by Erythrosin B stain exclusion.

The fusion technique used was modified from that of Gefter *et. al.,Somatic Cell Genetics,* 1977, *3*, 231, which is hereby incorporated by reference. Described below is the fusion experiment which yielded the hybridoma designated as H51C129. This hybridoma is deposited at the American Type Culture Collection (A.T.C.C.), 12301 Parklawn Drive, Rockville, MD, and is designated as A.T.C.C. Deposit No. HB 10538.

To a sterile 50 mL conical centrifuge tube was added $1\text{-}1.5\times10^8$ spleen cells with an equal number of SP 2/0 myeloma cells. The myeloma-spleen cell suspension was centrifuged at 1400 rpm for 5 minutes to pellet the cells together. The supernatant was aspirated off and the tube tapped gently to loosen the cell pellet and 1 mL of 50% polyethylene glycol (PEG, MW 1000, Sigma) in DMEM, without serum, was added to the cell pellet. The cells were resuspended gently in PEG solution over a period of 1 minute by slowly aspirating up and down using a 1 mL pipette. The tube was held in the hand for an additional 1 minute and then 1 mL of medium A was added slowly to dilute the PEG. The cells are allowed to stand for an additional 1 minute without agitation or mixing. An additional 20 mL of medium A was added over a period of 3 to 5 minutes, and the cells pelleted at 1400 rpm for 5 minutes. The supernatant was aspirated off and the cells resuspended in 20 mL of medium A with 20% fetal calf serum, 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 3×10^{-6} M thymidine (medium C or HAT selective medium).

Example 69

Selection of Hybridomas Producing Monoclonal Antibodies to PCB Immunogen of Example 17

The cell suspension from example 1 above was transferred into a 75 cm^2 T-flask and incubated at 37 °C In a 5% CO_2 incubator for 1-3 hours. The cell suspension was then diluted to 1×10^6 spleen cells/mL with medium C, and 1 mL volumes of the cell suspensions were added to each well of a 24-well Costar plates. These plates were incubated for 24 hours at 37 °C and 5% CO_2. After the incubation period 1 mL volumes of feeder cell (non-immunized BALB/c mouse spleen cells) suspension in medium C at $2\text{-}3\times10^5$ cells/mL was added to each of the 24 wells of the Costar plates and incubated at 37 °C, 5% CO_2 for 14-17

days. During this period, on alternate days, 1 mL volumes of medium is removed from each well by aspiration and replaced with 1 mL of fresh medium C. On day 10 the supernatants from the hybridoma containing wells were tested for antibody activity in the TDx instrument using selected tracers of EXAMPLE 26 and EXAMPLE 29, fenclofenac at 10% solution and 25 µL of hybridoma supernatant. Five hybridoma suspensions were chosen for further cloning by picking those supernatants with tracer binding in mP units greater than 20% over background. The cells from wells chosen for containing antibody activity were cloned by limiting dilution within 24 hours of sampling.

Example 70

Cloning of Hybridoma Culture that Produces Monoclonal Antibodies to PCBs

The cells in antibody secreting wells were diluted in a volume of medium B by a limiting dilution method to a concentration of 10 cells/mL. 100 µL of each diluted cell suspension were aliquoted into the wells of three 96-well Costar plates. 100 µL volumes of feeder cells in medium B at $5x10^5$ cells/mL were added to each well and the plates incubated at 37 °C, 5% CO_2 for 14 days. Supernatants were again tested for antibody activity using the same protocol as in EXAMPLE 69. The antibody producing clones were then expanded without feeder cells in 24 well Costar plates and finally in 25 cm^2 T-flasks. $32x10^6$ cells/mL samples of the clone were then stored in medium B with 10% glycerol added, in liquid nitrogen. 1-2 mL samples were then further evaluated for displacement on the TD_x instrument protocol and one clone (H51C129) was selected for ascites production.

Example 71

In Vivo Production of Monoclonal Antibodies to Example 70

An *in vivo* method for obtaining large amounts of monoclonal antibodies involved the adaptation of EXAMPLE 70 (hybridoma H51C129) to grow as an "ascites" tumor. Female BALB/c mice were printed by intraperitoneal injection of 0.5 mL of pristane (2,6,10,14-tetra-methylpentadecane). Approximately 4-5 weeks following the pristane injection, aliquots containing 1.5 x 10^6 actively growing hybridoma cells harvested from *in vitro* cultures as described in EXAMPLE 69 were innoculated into the peritoneal cavities of primed mice. Seven days following hybridoma cell injection, 5 - 10 mL of ascites fluid was harvested from each mouse. Upon purification by ammonium sulfate precipitation, approximately 24.6 mg of antibody was obtained per mL of ascites fluid.

Example 72

PCB Fluorescence Polarization Immunoassays

As described previously, the reagents for the FPIA of the present invention comprise tracers and antibodies raised against immunogens of the present invention, specific for PCBs. In addition, conventionally used assay solutions including a dilution buffer, and PCBs calibrators and controls are prepared. The preferred procedure was designed to be used in conjunction with an automated instrument such as Abbott Laboratories' TD_x, AD_x, or IM_x systems; however, manual assays can also be performed. In both procedures, the test sample can be mixed with a pretreatment solution and antibody in dilution buffer before a background reading is taken. The tracer is then added to the test solution. After incubation, a fluorescence polarization reading is taken.

The following extraction protocol was used to extract the non-polar PCBs into a water miscible solvent that could then be used in the automated assay on the TD_x instrument. 1 mL of standard or test sample was added to 9 mL of 10% acetone/hexane; mixed vigorously for one minute; sonicated in a water bath for 10 min. and centrifuged for 10 min at 1500 G. Then, 6 mL of hexane/acetone was removed and added to a 0.5 mL bed of propylene glycol. Hexane/acetone was evaporated off at 60 °C for 15 minutes under a gentle stream of air. After vigorous mixing, the mixture was sonicated for 10 minutes and centrifuged for 10 minutes at 1500 G. Following this, 0.5 mL TD_x buffer was added to the mixture; mixed vigorously; sonicated 10 minutes and centrifuged for 5 minutes at 10k/G. The solution from step 3 above was added to a sample well of a TD_x instrument and run via an automated assay protocol.

In the automated assays, the fluorescence polarization value of each calibrator, control or test sample was determined and printed on the output tape of the TD_x, AD_x or IM_x instrument. The instrument also

generated a standard curve by plotting the polarization of each calibrator versus its concentration, using a nonlinear regression analysis. The concentration of each control or sample was read off the stored curve and printed on the output tape.

The following reagents were used in the preferred automated PCB assays; one assay consisted of an antibody and tracer combination to bind and displace aroclors with a high concentration of highly chlorinated biphenyls, such as aroclors 1260 and 1254; another assay consisted of an antibody and tracer combination to bind and displace aroclors with a high concentration of comparably lower chlorinated biphenyls, such as aroclors 1016,1221,1232,1242 and 1248:

1) the pretreatment solution comprising 10% fenclofenac in 30 mM NaOH in water;

2) the respective assay's tracers diluted in 50% methanol in potassium phosphate buffer (0.15 M phosphate buffer, pH 7.5).

3) the respective assay's antibody comprising rabbit antisera or mouse monoclonal antibody raised against a PCB immunogen, diluted in TD_x buffer (0.1 M phosphate buffer, pH 7.5, containing 0.01% bovine gamma globulin and 0.1% sodium azide) with 30% glycerol;

4) a diluent buffer comprising TD_x buffer;

5) two sets of calibrators comprising 10% acetone in hexane containing 0.00, 1.0, 5.0, 15, 30, and 60 µg/mL of PCB aroclors 1221 and 1260;

6) controls comprising 5 µg/mL of aroclors 1221, 1254 and 1260.

All polarized fluorescent measurements were made using the TD_x instrument which performed the assay in accordance with the following protocol:

1) 22.5 µL of standard or test sample, and 12.5 µL each of the antibody reagent and the pretreatment reagent were delivered into a cuvette. A sufficient volume of diluent buffer was added to raise the volume to 1 mL, and a background intensity reading was taken;

2) 12.5 µL each of pretreatment reagent and antibody, 25 µL of the tracer, and the second 22.5 µL of sample and were added to the cuvette, and a sufficient volume of diluent buffer was added to raise the volume to 2.0 mL;

3) the reaction mixture was incubated;

4) the fluorescence polarization due to tracer binding to the antibody was obtained by subtracting the polarized fluorescence intensities of the background from the final polarized fluorescence intensities of the mixture; and

5) the polarization value for the unknown test sample was compared to a standard curve prepared using calibrators of known PCB content.

Example 73

FPIA

Data obtained from an immunoassay according to the present invention are summarized herein. The binding of tracer to antibody and the displacement of the tracer by the PCB present in the sample are summarized in Table 1 below. Various combinations of antibodies developed in response to immunogens and tracers, as described in the Examples above, were tested. In each combination where the tracer bound to the antibody, the net polarization was at least 150 millipolarization units, the 5 µg/mL span was at least 15 millipolarization units, and the intensity ratio varied between three and ten times that of the background noise.

As the data from Table 1 exemplify, the combination of the antibody produced by the immunogen of EXAMPLE 16 and the tracer of Example 26 provided good binding to the antibody and good displacement by PCB aroclors 1260 and 1254. The combination of the polyclonal or monoclonal antibody produced by the immunogen of EXAMPLE 17 and the tracer of EXAMPLE 29 provided good binding to the antibody and good displacement by PCB aroclors 1016, 1221, 1232, 1242, 1248 and 1254.

TABLE 1
(net mP)

Immunogen Example #	Tracer Example # RABBIT #(dil)	24	25	26	27	28	29	30	31	32	37	38	39	40	41	42	43	44
	Normal rabbit(1:1800)	100	103	105	74	72	79		43	43	61	70	42	51	41	32	56	57
18	4757(1:1800)	74	84	129	71	72	75		-	319	47	60	48	64	224	249	214	235
18	4758(1:1800)	80	134	279	65	69	76		207	324	55	62	44	56	278	238	246	269
18	4760(1:1800)	95	217	275	77	75	87		302	289	65	68	57	65	265	247	-	246
16	5272(1:1800)	129	200	314	75	58	82		-	334	54	50	46	56	215	197	195	208
16	5273(1:1800)	157	241	259	76	70	84		-	231	47	59	48	53	202	150	-	165
16	5274(1:1800)	247	287	239	73	69	75		-	170	39	59	43	53	245	198	-	216
17	5360(1:1800)	101	116	114	297	207	239		-	44	258	164	242	216	34	32	42	55
17	5361(1:1800)	99	112	109	294	224	243		-	43	261	170	257	228	36	32	44	53
17	5362(1:1800)	106	124	120	290	182	204		-	64	258	179	222	205	47	44	53	64

	Tracer Example #	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
	Normal rabbit(1:200)	78	94	71	40	115	87	117	98	59	36	79	141						
18	4757(1:200)	89	106	79	50	122	93	128	112	63	40	114	50						
18	4758(1:200)	78	105	95	61	127	126	123	112	58	46	122	162						
18	4760(1:200)	92	97	84	42	124	102	122	118	73	44	117	207						
16	5272(1:200)	90	109	178	47	136	108	135	216	168	86	136	-						
16	5273(1:200)	90	109	94	43	138	105	121	133	118	60	123	196						
16	5274(1:200)	102	118	95	45	129	111	126	136	77	37	119	172						
17	5360(1:200)	96	104	88	43	132	106	149	124	80	50	126	184						
17	5361(1:200)	86	114	83	42	128	106	153	175	92	56	131	193						
17	5362(1:200)	—	132	141	71	144	132	138	133	80	50	131	185						

	Tracer Example #	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
21	6033(1:1400)							224	184	26	181	161	231	24	180	144	77	196	268
21	6034(1:1400)							214	164	28	122	158	198	23	182	160	82	175	287
21	6035(1:1400)							205	155	27	113	158	217	24	102	133	40	174	212
20	6036(1:1400)																		
20	6037(1:1400)																		
20	6038(1:1400)																		
19	6039(1:1400)	187	185	231	216	176	240												
19	6044(1:1400)	192	177	228	221	151	216												
19	6046(1:1400)	202	178	223	212	150	233												

Table 2

5 µg/mL Spans

Immunogen:	EXAMPLE 17		EXAMPLE 17		EXAMPLE 16	
Antisera:	monoclonal		polyclonal		polyclonal	
Tracer:	EXAMPLE 29		EXAMPLE 29		EXAMPLE 26	
Aroclor	mP	span	mP	span	mP	span
0 control	218	--	192	--	216	--
1260	217	1	178	14	175	41
1254	176	42	112	80	137	79
1248	157	61	111	81	194	22
1242	149	69	96	96	199	17
1232	161	57	92	100	203	13
1221	165	53	109	83	212	4
1016	142	76	92	100	208	8

For each assay system, the binding of tracer to antibody and displacement of tracer by each aroclor mixture in the sample are summarized in Table 2 above.

Example 74

The cross-reactivity of a variety of structurally similar compounds was tested, and is as summarized in Table 3 below. Compounds were assayed by adding a known quantity of the test compound to PCB free propylene glycol, diluting to 50% solution in TDx buffer, and assaying the test samples on the TD_x instrument. The compounds were tested at a concentration of 100 µg/mL. The antisera or monoclonal antibody produced to the immunogens of the present invention, was determined to be highly specific to PCBs containing aroclors which in combination with the tracers of the present invention provide a sensitive FPIA for PCBs and is demonstrated by the data of Table 3.

Table 3
FPIA Specificity *

Immunogen:	EXAMPLE 16	EXAMPLE 17	
Antisera:	polyclonal	polyclonal	monoclonal
Tracer:	EXAMPLE 26	EXAMPLE 29	EXAMPLE 29
Test Compound			
β-BHC	0.0	0.018	0.0
α-BHC	0.0	0.0	0.0
γ-BHC	0.0	0.0	0.0
δ-BHC	0.0	0.0	0.0
toxaphene	0.0	0.0	0.0
endosulfan I	0.0	0.014	0.0
endosulfan II	0.0	0.0	0.0
endrin	0.013	0.0	0.0
endosulfan sulfate	0.0	0.0	0.0
heptachlor	0.0	0.0	0.0
heptachlor epoxide	0.0	0.0	0.0
dieldrin	0.0	0.0	0.0
4,4'-DDE	0.0	0.0	0.0
4,4'-DDD	0.0	0.0	0.0
1,2,4-trichlorobenzene	0.15	0.023	0.8
1,2 dichlorobenzene	0.0	0.0	0.0
chlorobenzene	0.0	0.0	0.0
2,5 dichlorophenol	0.0	0.0	0.0
3,4 dichlorophenol	0.0	0.0	0.0
pentachlorophenol	0.0	0.0	0.0
2,4 dichlorophenol	0.0	0.0	0.0
biphenyl	0.0	0.0	0.0
chlordane	0.018	0.0	0.15
4,4'-DDT	0.0	0.0	0.0
Example 12B	--	--	3.58
Example 11B	--	--	0.0
Example 13B	--	--	0.0

* units = μg/mL

Example 75

Fenclofenac was utilized in the assay system. The addition of fenclofenac to the assay minimizes the effect of any non-specific binding of the PCB and PCB tracers to serum proteins present in the assay system such as are found in the dilution buffer, antibody reagent or from the test sample. The final concentration of fenclofenac can range from about 0.005% to about 0.26% , preferably from about 0.02% to about 0.25%, with a concentration of about 0.13% being most preferred.

An experiment was conducted wherein fenclofenac was added to the sample pretreatment reagent

used. Fenclofenac was added at concentrations of 0%, 2% and 10% solutions and tested against tracer EXAMPLES 28 , 29, 31, and 32 in combination with normal rabbit serum which had not been innoculated with PCB immunogens and antisera Example 68 (immunogens from Examples 16, 17,and 18.) As the data of Table 4 exemplify, the addition of fenclofenac allowed specific antibody binding providing for acceptable net millipolarization and span.

Table 4

Tracers: (units = mP)		EXAMPLE 28	EXAMPLE 29	EXAMPLE 31	EXAMPLE 32
		(without fenclofenac)		(without fenclofenac)	
Normal Rabbit Serum 1:4		>300	>300	>300	>300
		2% fenclofenac		10% fenclofenac	
TDx Buffer		28	42	23	26
Normal Rabbit Serum 1:4		49	60	37	46
Antisera (polyclonal) 1:4		(Example 68)			
Example 18	4757	53	65	87	327
(immunogen)	4758	52	65	196	--
	4760	55	72	293	298
Example 16	5360	228	276	50	52
(immunogen)	5361	265	276	47	52
	5362	256	266	50	51
Example 17	5272	55	69	100	340
(immunogen)	5273	53	65	55	58
	5274	51	63	88	190

Example 76

Optionally, a PCB assay can be configured which utilizes a mixture of antibodies of EXAMPLE 16 and EXAMPLE 17, in combination with a mixture of tracers of EXAMPLE 29 and EXAMPLE 26 to assay all PCB aroclor mixtures in a single assay. The concentration ratios of the mixtures of antibodies and combined with mixtures of tracers can range from 10% to 90% of one antibody or tracer mixed with the other antibody or tracer type, with a mixture concentration ratio of about 3.5 parts antibody EXAMPLE 16 mixed with 1 part of antibody EXAMPLE 17, and 3.5 parts tracer EXAMPLE 26 mixed with 1 part of tracer EXAMPLE 29 being most preferred.

An experiment was conducted wherein 5 µg/mL of aroclor mixtures for 1260, 1254, 1248, 1242, 1232, 1221 and 1016 were assayed in the preferred one assay, mixed reagent system. As summarized in Table 5, the mixed reagent system provided an assay with an acceptable net millipolarization value and span.

Table 5
Mixed Reagent Assay

aroclor	mP	span
0	227	--
1260	209	19
1254	207	21
1248	208	20
1242	207	21
1232	207	21
1221	210	18
1016	211	17

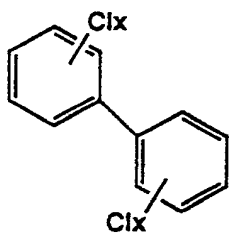
The incubation time for the assay can range from about 5 seconds to about 30 minutes. Preferably, the incubation time will be about less than five minutes. The incubation temperature also can vary between approximately ambient room temperature (25° C) and approximately 42° C; the preferred temperature is approximately 37° C. Incubation conditions and times can be varied by the routineer.

It will appreciated by those skilled in the art that many of the concepts of the present invention are equally applicable to other types of binding assays. The embodiments described and presented are intended as examples rather than as limitations. Thus, the description of the invention is not intended to limit the invention to the particular embodiments disclosed, but it is intended to encompass all equivalents and subject matter within the spirit and scope of the invention as described above and as set forth in the following claims.

Claims

1. A method for detecting the presence or amount of an analyte comprising polychorinated biphenyl in a test sample, which method comprises the steps of:
   a. adding a known concentration of a tracer labeled with a detectable moiety and a known concentration of an analyte-specific antibody to the test sample to form a mixture;
   b. incubating said mixture under conditions and for a time sufficient to form labeled tracer-antibody and analyte-antibody complexes; and
   c. determining the presence or amount of tracer-antibody complexes formed as a measure of the presence or amount of analyte in the test sample.

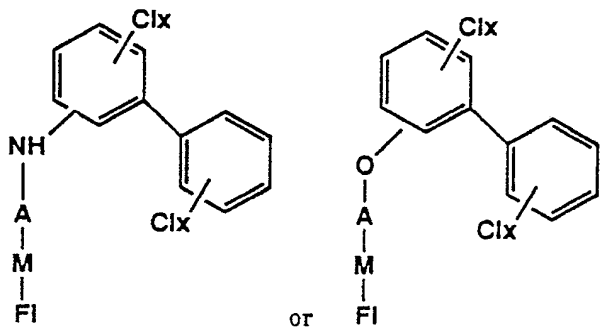
2. The method according to claim 1 wherein said polychlorinated biphenyls comprise the following structure



wherein x = 0-5.

3. The method according to claim 1 wherein the detectable moiety is selected from the group consisting of fluorescein and fluorescein derivatives and wherein said fluorescein derivatives are selected from the group consisting of fluorescein amine, carboxyfluorescein, α-iodoacetamidofluorescein, 4'-aminomethyl-fluorescein, 4'-N-alkylaminomethylfluorescein, 5-aminomethylfluorescein, 6-aminomethylfluorescein, 2-4-dichloro-1,3,5-triazin-2-yl-aminofluorescein, 4-chloro-6-methoxy-1,3,5-triazin-2yl-aminofluorescein and fluorescein isothiocyanate.

4. The method according to claim 1 wherein said tracer has a general structure of

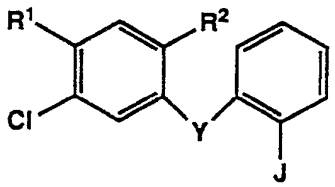


wherein

A is a spacer group consisting of from 0 to 50 carbons and heteroatoms, including not more than ten heteroatoms, arranged in a straight or branched chain, saturated or unsaturated, wherein not more than two heteroatoms are linked in sequence and branching occurs only on carbon atoms; and

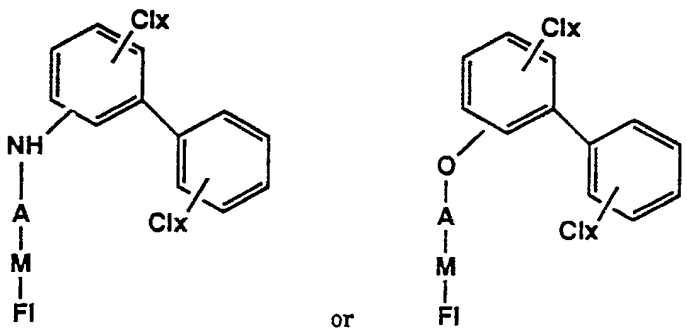
M is a linking group selected from the group consisting of >C=O, -NH-, O-C=O, N-C=O, N-C=S; and Fl is a detectable moiety.

5. The method according to claim 1 wherein step (a) further comprises adding to the test sample to prevent non-specific binding to proteins, a compound of the following structure



wherein Y = O, R^1 = Cl, R^2 = H and J = CH_2CO_2H.

6. A tracer of the general structure



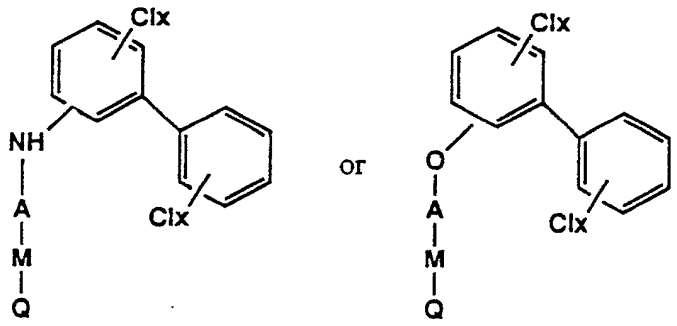
wherein

A is a spacer group consisting of from 0 to 50 carbons and heteroatoms, including not more than ten heteroatoms, arranged in a straight or branched chain, saturated or unsaturated, wherein not more than two heteroatoms are linked in sequence and branching occurs only on carbon atoms; and

M is a linking group selected from the group consisting of >C=O, -NH-, O-C=O, N-C=O, N-C=S; and

Fl is a detectable moiety.

7. An immunogen having the general structure



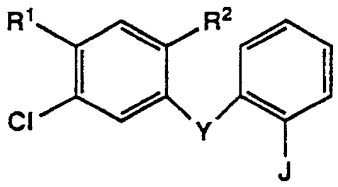
A is a spacer group consisting of from 0 to 50 carbons and heteroatoms, including not more than ten heteroatoms, arranged in a straight or branched chain, saturated or unsaturated, wherein not more than two heteroatoms are linked in sequence and branching occurs only on carbon atoms; and

M is a linking group selected from the group consisting of >C=O, -NH-, O-C=O, N-C=O, N-C=S; and

Q is an immunogenic carrier.

8. The immunogen according to claim 7 wherein said immunogenic carrier is selected from the group consisting of albumin, serum proteins, olular lens proteins, lipoproteins, bovine serum albumin, keyhole limpet hemocyanin, egg ovalbumin, thyroglobulin and bovine gamma globulin.

9. An additive compound for reducing non-specific binding of polychlorinated biphenyls and tracers to proteins and surfaces having the structure



wherein Y=O, R^1=Cl, R^2=H and J=CH_2CO_2H.

10. A kit for detecting the presence or amount of an analyte comprising polychorinated biphenyl in a test sample which kit comprises:

a. an analyte-specific antibody;

b. a tracer labeled with a detectable moiety; and

c. an additive compound which prevents non-specific binding of the analyte to proteins.

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(54) **Reagents and method for detecting polychlorinated biphenyls.**

(57) Reagents and an immunoassay for detecting the presence or amount of polychlorinated biphenyls in a test sample. The assay is performed by adding a known concentration of a tracer labeled with a detectable moiety and a known concentration of an analyte-specific antibody to a test sample to form a mixture, incubating the mixture to form labeled tracer-antibody and analyte-antibody complexes, and determining the presence or amount of tracer-antibody complexes formed as a measure of the presence or amount of analyte in the test sample. Reagents provided include tracers, immunogens and an additive compound useful in preventing non-specific binding of the polychlorinated biphenyls to proteins which may be present in the test sample. A kit for performing the assay also is provided.

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X,D	US-A-4 456 691 (S. STARK) * examples III-IV * ---	1,2,6-8	G01N33/53 G01N33/531 G01N33/577
A	CHEMOSPHERE vol. 16, no. 8-9, 1987, LONDON UK pages 1635 - 1639 L. STARKER ET AL. 'development of an immunoassay for chlorinated dioxins based on a monoclonal antibody and an enzyme linked immunosorbent assay (ELISA).' * the whole document * -----	1-10	C12P21/00 C07K15/00 C07C25/18 G01N33/532 G01N33/533

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

G01N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	20 JANUARY 1993	VAN BOHEMEN C.G.

CATEGORY OF CITED DOCUMENTS

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